The Chemical And Physical Characterisation Of Illicit Tablets And Development Of A Statistical Model To Identify Populations Within The Illegal Supply Chain

A thesis submitted for the degree of Doctor of Philosophy (PhD)

by

Mavis Ann MacDougall-Heasman

School of Science, Engineering and Technology,
Abertay University.

October, 2018

Declaration

Candidate's declarations:

I, Mavis Ann MacDougall-Heasman, hereby certify that this thesis submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy (PhD), Abertay University, is wholly my own work unless otherwise referenced or acknowledged. This work has not been submitted for any other qualification at any other academic institution.

Signed [candidates signature]... [redacted] .....

Date..... 17th October 2018 .....

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I, Isobel Stewart, hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy (PhD) in Abertay University and that the candidate is qualified to submit this thesis in application for that degree.

Signed [Principal Supervisors signature]..... [redacted]

Date..... 17th October 2018 .....

Certificate of Approval

I certify that this is a true and accurate version of the thesis approved by the examiners, and that all relevant ordinance regulations have been fulfilled.

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Acknowledgements

I would like to thank my supervisors, Mrs Isobel Stewart, Dr Anne Savage and Prof. David Bremner for their guidance, support, advice and endless patience throughout my project. Their encouragement and wisdom has given me the opportunity to achieve more than I had imagined both in terms of carrying out the research and in presenting at various international conferences, for which I am deeply grateful.

My thanks also go to the technical staff at Abertay and in particular Ms Louise Milne and Mr Maurice Lindsay, whose knowledge, advice and support has been invaluable. The lessons in how to strip down and clean out the HPLC were helpful for practical reasons and to help me think through processes more logically.

I would also like to thank the staff at Robert Gordon University, particularly Dr Ann Tough and Mr. Iain Tough for allowing me access to their valuable resources and providing the necessary training. Special thanks to Dr Kerr Matthews for his support, guidance and expertise, as well as indulging my interest to test baby milk both by DSC and in the tablet press. The lessons on thermal analysis and tablet production were invaluable. Thanks also to Ms Shahdia Bibi, Dr Laurie Smith and Mr Stephen Williamson for their endless support.

Thank you also to Dr Nia White and Mrs Wendy Robb in the Graduate School for their guidance and encouragement during my research period and, to Abertay University RLincs project for the financial support for the research.

Dedication

For my mum, my dad and Richard for supporting me. For Jordan, Marietta and Brooke for believing in me.

For Mairi for always being there and, for Helen because nothing is forgotten. Nothing is ever forgotten.

Abstract

Diazepam is an effective pharmaceutical used for both medicinal and illicit purposes and its popularity has led to the sale of both diverted pharmaceutical tablets and illicitly manufactured products. The aim of this project was to characterise 65 different cases of illicit tablets seized from the Tayside area. Physical and chemical analysis was compared with known pharmaceutical tablets and resulting data was statistically evaluated, to reveal potential links between illicit cases and distinguish pharmaceutically manufactured diverted products.

Physical differences between cases revealed many of the tablets were not pharmaceutically manufactured for the UK market. This was supported by chemical analysis using GC-MS and HPLC, which indicated that only 39 of the 63 cases analysed contained diazepam as the main active ingredient, with diazepam levels varying between 8 – 48 mg and more potent substances found in many tablets. An innovative use of DSC demonstrated great sensitivity in distinguishing between cases, based largely on excipients. A novel approach for forensic analysis was taken both through visual comparison of thermograms and by statistical analysis of resulting data.

The statistical clustering techniques of AHC and k-means were used to explore combined results and potential links between a subset of cases were visualised in a heat map. DSC data points were then explored by PCA and LDA to further distinguish between the pharmaceutical tablets and seized cases.

Overall, a combination of physical characteristics, chemical properties and statistical analysis were able to distinguish between the small number of pharmaceutically manufactured diverted cases and the majority revealed to be illicitly produced. This is important new research into street drugs, which reveals an insight into the illicit market and the dangerous nature of blue tablets being sold, providing valuable information to both police and medical services.

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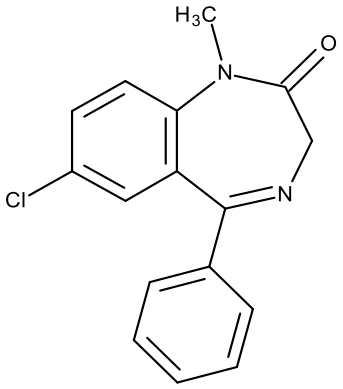
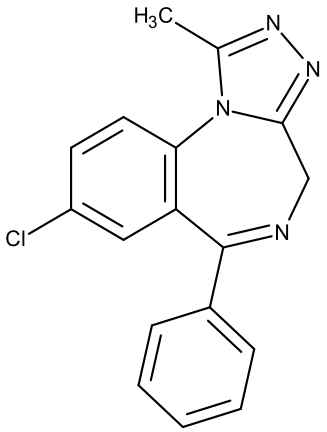
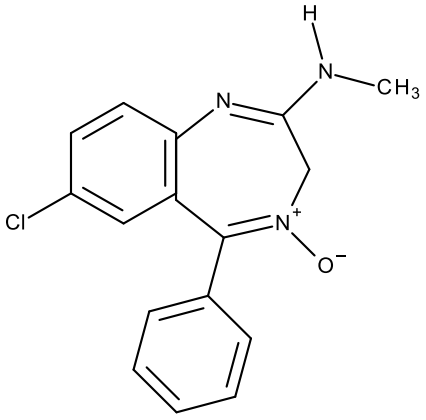
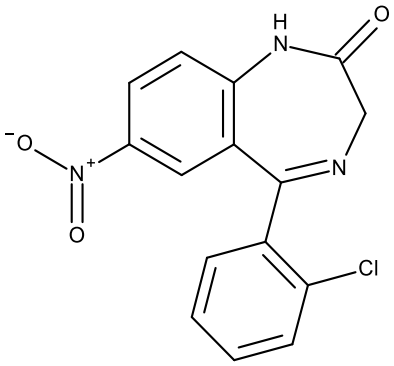
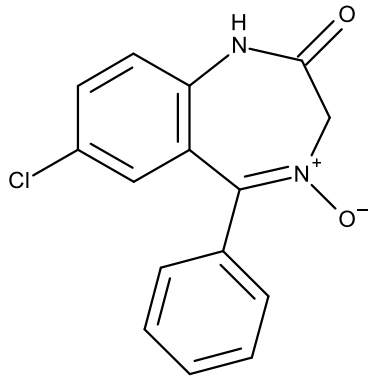
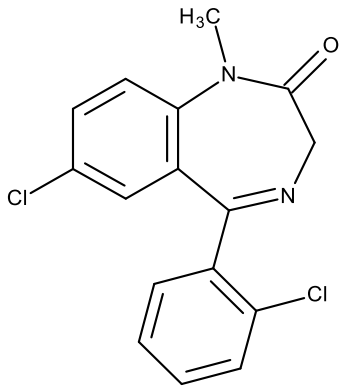
List of Abbreviations

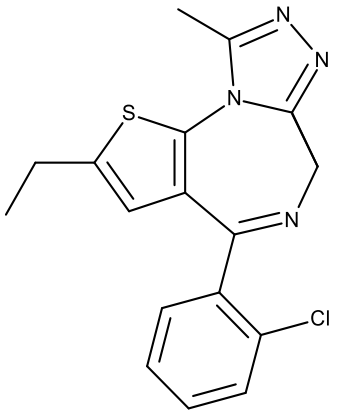
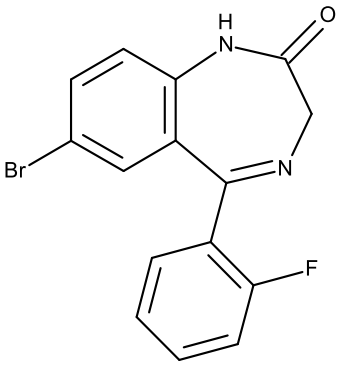
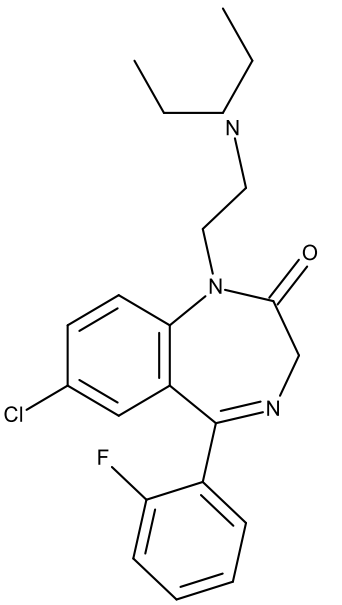
AC_2O	Acetic Anhydride
AMU	Atomic Mass Unit
AHC	Agglomerative Hierarchical Clustering
^oC	Degrees Celsius
CDA	Canonical Discriminant Analysis
CI	Chemical Ionisation
CNS	Central Nervous System
CT	Computerized Tomography
CZE	Capillary Zone Electrophoresis
D	Discriminant Value
DART-MS	Direct Analysis in Real Time - Mass Spectrometry
Dpi	Dots per inch
DSC	Differential Scanning Calorimeter
DFA	Discriminant Function Analysis
DFC	Discriminant Function Coefficient
DSLR	Digital Single Lens Reflex
EDX	Energy Dispersive X-Ray
EI	Electron Ionisation
ELISA	Enzyme-Linked Immunosorbent Assay
EtOAc	Ethyl Acetate
GABA	Gamma-Amino Butyric Acid
GC-MS	Gas Chromatography Mass Spectrometry
HPLC	High Performance Liquid Chromatography
ISO	International Standards Organisation
ITMS	Ion Trap Mass Spectrometer
LC	Liquid Chromatography
LDA	Linear Discriminant Analysis
LOD	Limit of Detection
LOOCV	Leave-One-Out Cross Validation
LOQ	Limit of Quantification

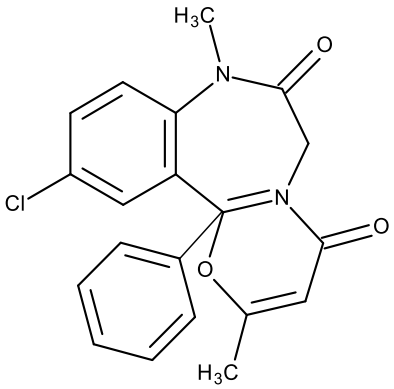
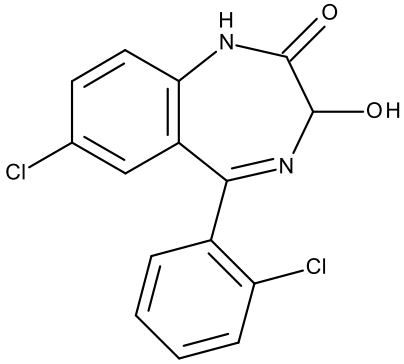
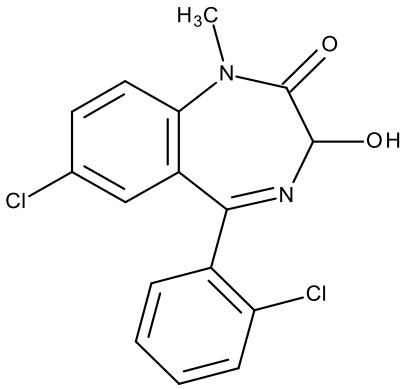
M	Molarity
MA	Methamphetamine
mAUs	Milli-Absorbance Units
MDA	3,4-Methylenedioxyamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
MHRA	Medicines and Healthcare products Regulatory Agency
ms	Mass Spectrum
MS	Mass Spectroscopy
MS/MS	Tandem Mass Spectroscopy
m/z	Mass to Charge Ratio
NIR	Near Infrared
NIST	National Institute of Standards and Technology
PCA	Principal Component Analysis
QMS	Quadrupole Mass Spectrometer
ROC	Receiver Operating Characteristic
RSD	Relative Standard Deviation
R_t	Retention Time
SD	Standard Deviation
SEM	Scanning Electron Microscope
TDGC	Thermal Desorption Gas Chromatography
TIC	Total Ion Chromatogram
TLC	Thin Layer Chromatography
UK	United Kingdom
UNODC	United Nations Office on Drugs and Crime
USA	United States of America
UV	Ultraviolet
VSC	Video Spectral Comparator
WHO	World Health Organisation
Δ (Delta)	Heat

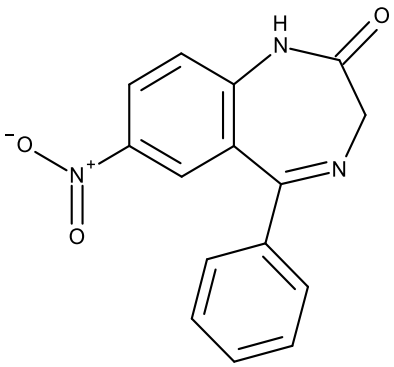
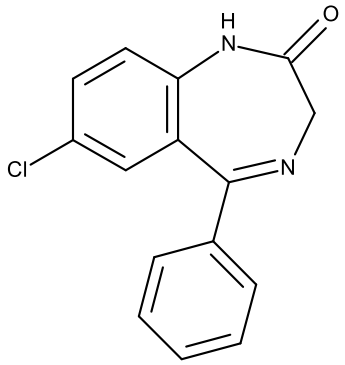
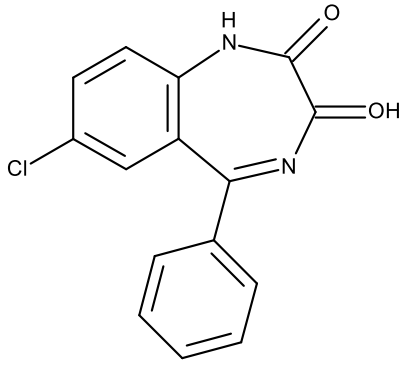
Benzodiazepine Structures referred to in this Thesis

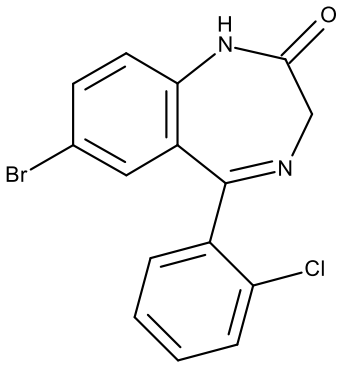
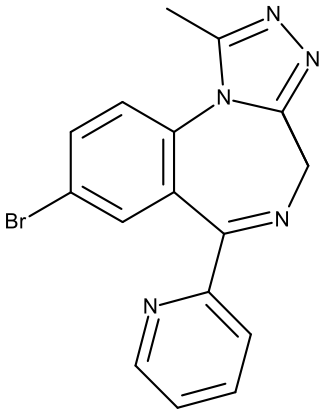
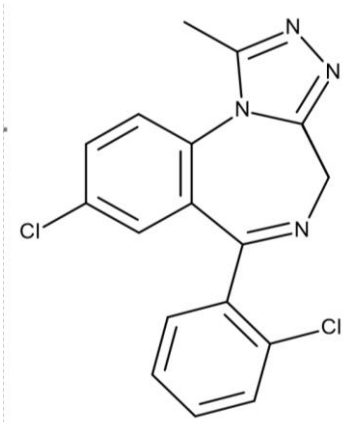
Structure Numer	Benzodiazepine Common and IUPAC Name	Structure
1	**Diazepam** 7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one	
2	**Alprazolam** 8-Chloro-1-methyl-6-phenyl-4H-s-triazolo(4,3-a)(1,4)benzodiazepine	
3	**Chlordiazepoxide** 7-chloro-4-hydroxy-N-methyl-5-phenyl-3H-1,4-benzodiazepin-2-imine	

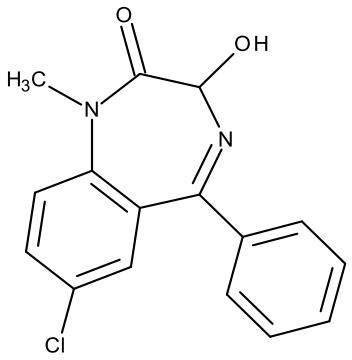
Structure Numer	Benzodiazepine Common and IUPAC Name	Structure
4	**Clonazepam** 5-(2-chlorophenyl)-7-nitro-1,3-dihydro-1,4-benzodiazepin-2-one	
5	**Demoxepam** 7-Chloro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepine-2-one-4-oxide	
6	**Diclazepam** 7-chloro-5-(2-chlorophenyl)-1-methyl-3H-1,4-benzodiazepin-2-one	

Structure Numer	Benzodiazepine Common and IUPAC Name	Structure
7	**Etizolam** 4-(2-Chlorophenyl)-2-ethyl-9-methyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine	
8	**Flubromazepam** 7-Bromo-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one	
9	**Flurazepam** 7-chloro-1-[2-(diethylamino)ethyl]-5-(2-fluorophenyl)-3H-1,4-benzodiazepin-2-one	

Structure Numer	Benzodiazepine Common and IUPAC Name	Structure
10	**Ketazolam** 11-Chloro-2,8-dimethyl-12b-phenyl-8,12b-dihydro-4H-[1,3]oxazino[3,2-d][1,4]benzodiazepine-4,7(6H)-dione	
11	**Lorazepam** 7-chloro-5-(2-chlorophenyl)-3-hydroxy-1,3-dihydro-1,4-benzodiazepin-2-one	
12	**Lormetazepam** 7-chloro-5-(2-chlorophenyl)-3-hydroxy-1-methyl-3H-1,4-benzodiazepin-2-one	

Structure Numer	Benzodiazepine Common and IUPAC Name	Structure
13	**Nitrazepam** 7-nitro-5-phenyl-1,3-dihydro-1,4-benzodiazepin-2-one	
14	**Nordiazepam** 7-chloro-5-phenyl-1,3-dihydro-1,4-benzodiazepin-2-one	
15	**Oxazepam** 7-chloro-3-hydroxy-5-phenyl-1,3-dihydro-1,4-benzodiazepin-2-one	

Structure Numer	Benzodiazepine Common and IUPAC Name	Structure
16	**Phenazepam** 7-bromo-5-(2-chlorophenyl)-1,3-dihydro-1,4-benzodiazepin-2-one	
17	**Pyrazolam** 8-bromo-1-methyl-6-pyridin-2-yl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine	
18	**Triazolam** 8-Chloro-6-(2-chlorophenyl)-1-methyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine	

Structure Numer	Benzodiazepine Common and IUPAC Name	Structure
19	**Temazepam** 7-chloro-3-hydroxy-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one	

Chapter 1. Aims and Objectives of this Study

1.1 Aims of the Research

Benzodiazepines have been implicated in an increasing number of drug related deaths in Scotland in recent years. The threat posed is elevated by the existence of illicitly manufactured tablets, which are in circulation within the illegal supply chain (Police Scotland, 2016; McGivern, 2016), with 10 mg tablets believed to be the most abused of the diazepam (**1**) tablets (Doward, 2012; McGivern, 2018).

The aim of this project was to characterise sixty-five different cases of illicit blue tablets, believed to have been sold as 10 mg diazepam (**1**) tablets, which had been seized in the Tayside area by Police Scotland. By measuring a range of physical and chemical characteristics, it was intended to generate data which could be tested using statistical clustering methods to determine whether these characteristics could be used to link separate cases. Principal component analysis and linear discriminant analysis of the dataset would then be used with the purpose of distinguishing known pharmaceutical tablets from pharmaceutically plausible tablets.

1.2 Objectives of the Research

The objectives of this thesis were to analyse the physical and chemical properties of seized cases of blue tablets, in order to characterise them. This included recording the weight, diameter and depth of the 1989 tablets contained within the sixty-five seized cases being assessed and the five batches of pharmaceutical tablets used for comparative purposes. Chemical analysis was performed using gas chromatography-mass spectrometry (GC-MS) to identify the main active drug substances and high performance liquid chromatography (HPLC) for quantification of diazepam (**1**) content. Information about the excipients was determined by differential scanning calorimetry (DSC).

All data on the physical and chemical properties of the tablets was stored in an MS Access database which could be searched and subsets extracted.

The first objective of the statistical analysis was to determine whether illicit cases could be linked by identifying clusters within the data derived from illicit tablets. Clustering was carried out using visual methods, agglomerative hierarchical clustering and k-means clustering.

The second objective of the statistical analysis was to use the physical and chemical data to develop a series of methods that could distinguish illicitly made tablets from possible pharmaceutical tablets that had been diverted into the illegal supply chain. Principal component analysis and linear discriminant analysis was used for further analysis of cases that most resembled the pharmaceutically manufactured tablets.

1.3 Chapter Overview

The work undertaken for this project will be described in detail in the following Chapters:-

Chapter 2 – Introduction

This chapter provides a background to this project and describes the diazepam (**1**) drug substance in context of the discovery and use of benzodiazepines. The increase in diazepam (**1**) use and abuse is explained with data showing the numbers of deaths related to its misuse and the UK legislation concerning benzodiazepines is also described.

Factors influencing the effectiveness of the drugs, including tablet manufacturing processes are explored. This chapter also exposes how illicit tablets can pose hidden dangers with their highly variable composition and how differences can be identified between tablets produced by different manufacturers.

Chapter 3 – Physical Characteristics

The techniques used to measure the physical weights and measurements of seized illicit tablets and those of pharmaceutical origin are described. The weight, diameter and depth of all tablets were recorded on a Microsoft access database, enabling comparisons to be performed. Data was analysed and visualised in graphical form and by using box plots.

The colour of the tablets was recorded by photography using a purpose built lightbox.

Chapter 4 – Gas Chromatography - Mass Spectrometry

Tablets from each of the cases were analysed by GC-MS in order to confirm the active drug substance present. Although the street samples were believed to be sold as 10 mg diazepam (**1**) tablets and were blue in colour, the origin of the tablets was unknown and therefore the actual content was also uncertain. However, tablets containing diazepam (**1**) were not necessarily of pharmaceutical origin.

Chapter 5 – High Performance Liquid Chromatography

HPLC was used to quantify the diazepam (**1**) content of three tablets taken from each case. All of the illicit cases analysed were blue in colour, which is consistent with the colour of 10 mg diazepam (**1**) tablets manufactured for the UK market. However, the presence of the correct amount of active drug substance does not guarantee the tablets have been pharmaceutically manufactured.

Chapter 6 – Differential Scanning Calorimetry

The thermal technique of differential scanning calorimetry was tested as a way to explore differences in the excipient content of the tablets. A large proportion of a pharmaceutical diazepam (**1**) tablet is comprised of excipients, with an anticipated amount of about 10 mg of active drug substance being present. Therefore, information gained on the excipient content was explored as a way to differentiate between different tablets.

Chapter 7 – Statistical Cluster Analysis

The information gained from the physical and chemical tests was used to provide data for statistical and chemometric analysis. Agglomerative hierarchical clustering and k-means clustering were used to explore patterns in the data and identify potential groupings. The results of all of the experiments were then added to a heat map under the premise that the more individual cases cluster with other individual

cases, as an indication of the probability of there being a potential link between them.

Chapter 8 – Linear Discriminant Analysis

In order to further examine the seized cases which contained approximately 10 mg of diazepam (**1**), a smaller test sample was chosen. As the tablets could not be differentiated from the pharmaceutical tablets based on physical characteristics or type and quantity of active drug substance, the data generated by DSC was analysed using chemometric methods. DSC was chosen because the thermograms generated were largely influenced by the excipient content of the tablets. The data points were explored by principal component analysis and linear discriminant analysis to determine if any differences could be found between the pharmaceutically manufactured tablets and the street diazepam (**1**) cases.

Chapter 9 – Conclusion and recommendations for Future Work

The conclusion describes the context in which this study was based and briefly summarises the work performed and results obtained.

Innovative methods of analysis such as the forensic use of the DSC performed during this project, are described along with its potential benefits. Ideas for future work are also discussed, in order to build upon the analysis performed during this project. The research carried out in this study has provided an important step on which to base future work and suggestions are described with a view to obtaining further information that would help in the characterisation of illicit tablets.

Appendices

The appendices contain “hand-worked” examples of the statistical techniques of principal component analysis, agglomerative hierarchical clustering and k-means clustering and the script used for principal component analysis using the ‘R’ programming system.

Chapter 2. Introduction

2.1 Chapter Summary

This chapter provides a background to the project and describes the diazepam (**1**) drug substance in context of the discovery and use of benzodiazepines. The increase in diazepam (**1**) use and abuse is explained with data showing the numbers of deaths related to its misuse and the UK legislation concerning benzodiazepines is also described.

Factors influencing the effectiveness of the drugs, including tablet manufacturing processes are explored and exposes how illicit tablets can pose hidden dangers with their highly variable composition and how differences can be identified between tablets produced by different manufacturers.

2.2 Diazepam

2.2.1 Description and Structure

Diazepam (**1)**, or 7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one is a benzodiazepine type drug with the chemical formula $C_{16}H_{13}ClN_2O$ (Figure 2.1). In its pure pharmaceutical form, diazepam (**1**) is odourless and has the appearance of an off-white crystalline powder (British Pharmacopoeia Commission, 2017a). The chemical structure includes a heterocyclic ring containing nitrogen, making it an alkaloid. Many alkaloids have been found to contain pharmacological properties (Aniszewski, 2015).

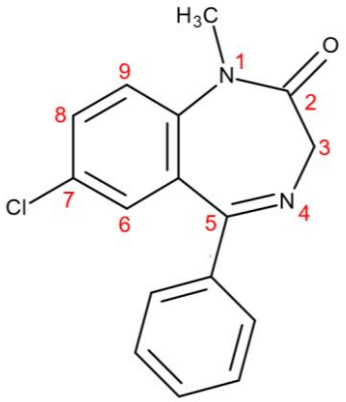


Figure 2.1. The structure of diazepam (1), with carbon numbering.

Medically, diazepam (**1**) is used to treat anxiety, sleep disorders, muscle spasms and alcohol withdrawal. However, over time there may be an increased tolerance to many of the sedative effects and both physical and psychological dependence can develop. Sudden withdrawal from diazepam (**1**) can cause adverse reactions including nausea, restlessness, anxiety, nervousness, tremors, dysphoria, insomnia, stiffening muscles, abdominal pain and headaches. In more extreme cases hallucination, hyperthermia, panic attacks, disorientation and delirium can set in, leading to tachycardia, hypertension and seizures (Ashton, 2013). These risks are aggravated by the use of other drugs including opiates, barbiturates, alcohol and other central nervous system depressants. Benzodiazepines are sedatives and taken with other sedatives, such as barbiturates, have an accumulative effect. Therefore dangerous doses of other substances could lead to fatality when consumed in conjunction with benzodiazepines (Medicines and Healthcare Products Regulatory Agency, 2015b; Ashton, 2013).

2.2.2 Discovery of Diazepam

Sedatives have long been used for medical purposes to aid physiological and mental depression, producing a more sedate state. Anxiolytic drugs are designed to reduce anxiety without affecting physical functions but many have sedative properties, leading to a degree of overlap between treatments (Rang, 2016). Previous sedatives have included alcohol, chloral hydrate and bromides but all of these have been shown to have serious side effects. Bromides for example, are toxic, with a half-life of approximately twelve days and accumulation in tissues, leads to delirium,

hallucinations, gastrointestinal problems and irritability (López-Muñoz, Álamo and García-García, 2011).

The synthesis of barbituric acid in 1864 gave rise to the introduction of the first barbiturates in 1904. Almost immediately problems with tolerance, dependence, ease of overdose and hazards of withdrawal were recorded. However, over 2500 barbiturates were synthesised by the early 1950s with around 50 introduced into clinical use (López-Muñoz, Ucha-Udabe and Alamo, 2005; Dundee and Mcilroy, 1982; Maxwell, 2012).

An alternative to the barbiturates, meprobamate was introduced as an anxiolytic in the 1950s but had limited effects, while dangers posed by tolerance, dependence, potential for abuse and overdose meant it was withdrawn in North America during the 1960s (López-Muñoz, Ucha-Udabe and Alamo, 2005). Interestingly, the last licence for meprobamate production in the United Kingdom was only withdrawn after a review across the European Union in 2016 (Medicines and Healthcare Products Regulatory Agency, 2016a).

During the 1950's the dangers posed by prescribed sedatives created an impetus for the development of new safer drugs. The intention to patent a new product inspired Hoffmann-La Roche to research compounds previously unused by the pharmaceutical industry (Weatherall, 1990). Leo Sternbach investigated what were thought to be benzheptoxdiazines but were later classified as quinazoline 3-oxides (Sternbach, 1979). Previous post-doctoral research into dyes had demonstrated that these compounds were easy to synthesize, crystallize, isolate and purify. The marketing of the antipsychotic drug chlorpromazine, with a chain attached to the tricyclic compound, encouraged Sternbach to investigate the effects of different side chains. Chlorpromazine is a phenothiazine derivative, comprising of a tricyclic compound substituted with chlorine at position 2 and tertiary alkylamine at position 10 (Figure 2.2).

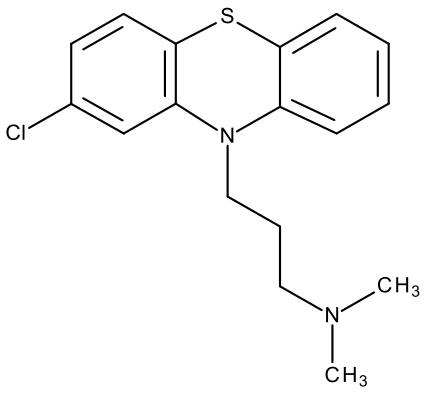


Figure 2.2. Structure of chlorpromazine.

Phenothiazines are biologically active and Sternbach decided to investigate whether altering the side chains could affect their biological influence (López-Muñoz, Álamo and García-García, 2011). Amino ketones with different benzene ring substituents were acylated and further treatment with secondary amines was performed to produce a variety of side chains. Hydrogenation and removal of the oxygen from the N-oxide were both attempted, however, laboratory research indicated that the compounds produced no pharmacological effect (Sternbach, 1979). Further work at Hoffmann-La Roche meant that the investigation into the compounds had to be postponed but eighteen months later, during a laboratory clean-up operation, a crystalline substance resulting from the earlier work was discovered and sent for biological assessment (Maxwell, 2012). The results indicated that the substance relaxed muscles and calmed mice by preventing convulsions caused by electrical stimuli; and monkeys were found to be less aggressive without apparent tiredness, after being treated with the drug. Toxicologically it was discovered that large quantities could be consumed without fatal consequences. The benefit being that in cases of overdose, the patient may become unconscious but would not manifest symptoms of asphyxiation or respiratory failure, which could occur with barbiturates, and it did not affect the heart (Weatherall, 1990; Maxwell, 2012). Another benefit was the greater difference in human dosage levels required between anxiolytic and sedative effects, when compared to barbiturate or meprobamate concentrations (Maxwell, 2012). The substance was marketed as Librium in 1960 and was given the generic name chlordiazepoxide (**3**) (Figure 2.3).

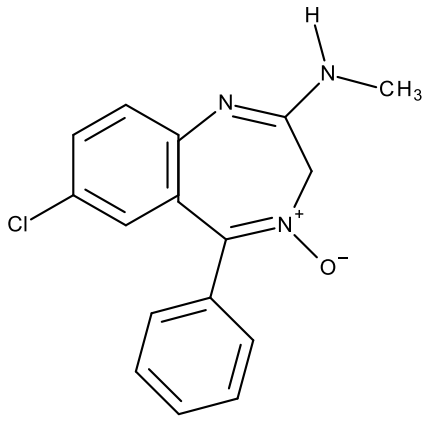


Figure 2.3. Structure of chlordiazepoxide (3).

Sternbach performed further research into chlordiazepoxide (**3**) in order to determine why this had been the only one of the synthesized substances to demonstrate physiological properties. The results indicated that a primary amine had been used instead of a secondary amine, during the final treatment, causing a ring enlargement reaction to occur (López-Muñoz, Álamo and García-García, 2011).

Chlordiazepoxide hydrochloride was found to be bitter, hygroscopic and unstable but examination of the main metabolite, demoxepam (**5**) revealed that simplification of the compound by removal of the N-oxide functionality resulted in a more potent product. This became the new parent compound and exploration began into the addition of chains in positions 1 and 3, as shown in Figure 2.4. The 1-methyl derivative proved to be more effective than chlordiazepoxide (**3**) and it was marketed as Valium (**1**) in 1963 (Sternbach, 1979; López-Muñoz, Álamo and García-García, 2011). When the patent expired in 1985, production was opened to generic forms of the drug which were termed diazepam (**1**) (Calcaterra and Barrow, 2014).

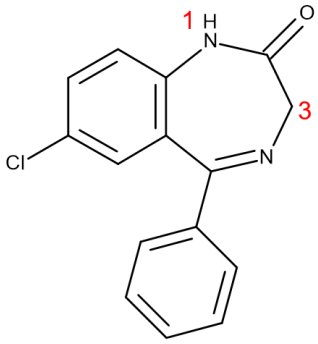


Figure 2.4. The chemical structure of the parent compound used in Sternbach's research. Positions 1 and 3, which are indicated in red, identify the locations where different groups were substituted. (Sternbach, 1979)

2.2.3 Benzodiazepine Family

Benzodiazepines are characterised by a benzene ring, which is fused to a seven-membered diazepine ring containing nitrogen at positions 1 and 4 (Sultan and El-Mubarak, 1996). Research by Sternbach revealed that the pharmacological effects increased when electron withdrawing groups, such as chlorine or nitro were attached to position 7 of Ring A, (Figure 2.5). Electron donating groups, such as a methyl, caused a reduction in pharmacological properties. Sternbach also discovered that groups located in any other position on Ring A, diminished pharmacological activity.

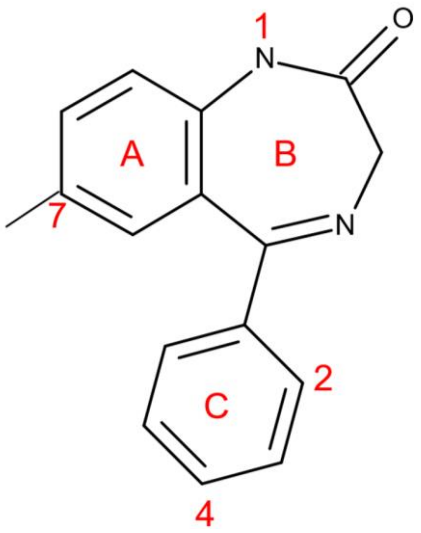


Figure 2.5. Structure used by Sternbach as a basis for studying the pharmacological effects of different substituents and their positions. (Sternbach, 1979)

The addition of a methyl group on position 1 of Ring B also produces an increase in pharmacological activity but larger groups can diminish the effect. The addition of a phenyl in position 5 also demonstrated greater biological effects. Pharmacological properties increase with a halogen in position 2 of Ring C, as demonstrated in phenazepam (**16**) (Figure 2.6) with a potency around five times that of diazepam (**1**) (World Health Organisation, 2015c). However, the pharmacological properties decrease when a group is added to position 4. During his studies on diazepam (**1**) compounds Sternbach synthesised over 3000 related compounds (Sternbach, 1979).

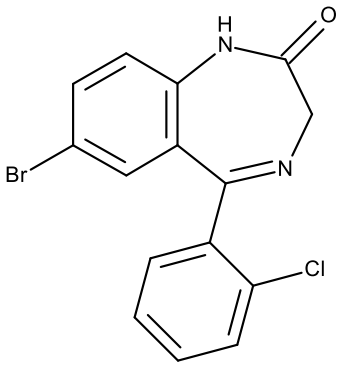


Figure 2.6. Structure of phenazepam (16).

After the launch of chlordiazepoxide (**3**) in 1960 and diazepam (**1**) in 1963 by Hoffmann-La Roche, the first benzodiazepine marketed by a different company was oxazepam (**15**), sold under the brand name Serax, by Wyeth laboratories in 1965. Research had indicated that the N-oxides could easily be synthesized and after acetic acid or acetic anhydride or treatment, a 3-acetoxy derivative is formed. The 3-hydroxy derivative (oxazepam **15**) is then obtained through hydrolysis (Figure 2.7) (Sternbach, 1979).

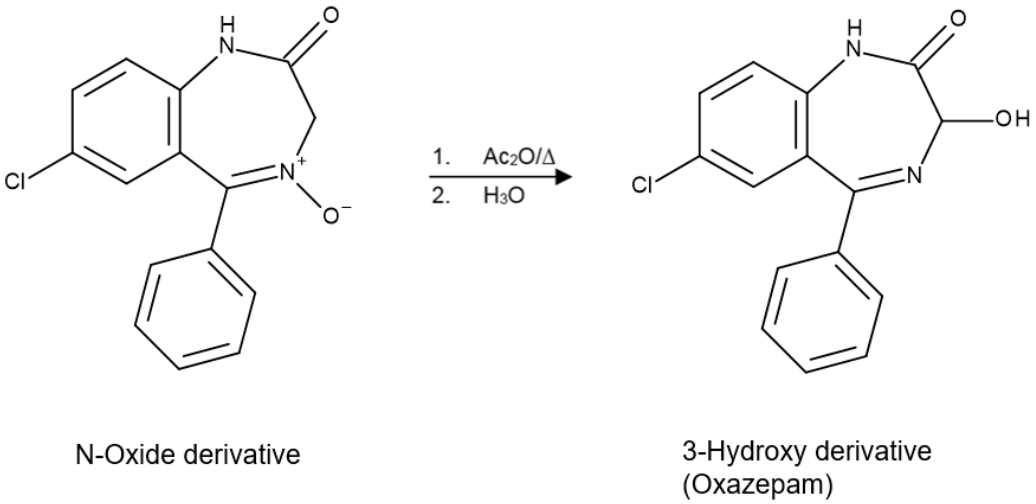


Figure 2.7. The synthesis of Oxazepam (15). (Sternbach, 1979)

Oxazepam (**15**) is an active metabolite of diazepam (**1**) and undergoes glucuronidation to aid excretion from the body. A potential disadvantage of being prescribed diazepam (**1**), is that the demethylation process results in the production of nordiazepam (**14**) (Figure 2.8), which has a much longer half-life of between 36-200 hours. This poses particular problems for the elderly as the oxidative reaction slows during the ageing process, thus increasing the time before converting to oxazepam (**15**) and then exiting the body (Ashton, 2013; Rang, 2016).

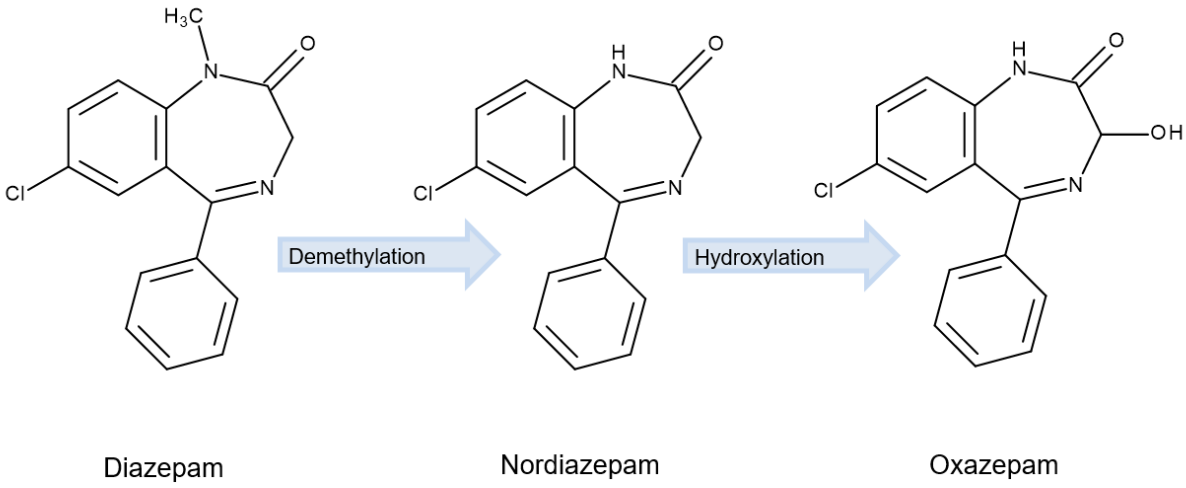


Figure 2.8. The metabolism of diazepam (1), showing the active metabolites nordiazepam (14) and oxazepam (15).

The degrees of pharmacological activity produced by the benzodiazepines vary with the drug substance, therefore a number of them are used for different clinical purposes. For example, diazepam (**1**), oxazepam (**15**) and chlordiazepoxide (**3**) are marketed as anxiolytics; clonazepam (**4**) as an anti-convulsant; and nitrazepam (**13**) as a hypnotic, to aid sleep. However, there may be some cross-over depending on the symptoms and the patient, so clonazepam (**4**) may also be used as an anxiolytic, for example and oxazepam (**15**) is prescribed for treating insomnia. The benzodiazepines also vary in absorption rate, active metabolites and length of duration (Ashton, 2013; Maxwell, 2012)

Potency varies between the different benzodiazepines. For example, a 10 mg dose of diazepam (**1**) would be equivalent to 25 mg of chlordiazepoxide (**3**), 20 mg of oxazepam (**15**), 2 mg of phenazepam (**16**) or 0.5 mg of alprazolam (**2**) (Ashton, 2013; World Health Organisation, 2015c). Each of these are prescribed as anxiolytics, however, it is the speed of onset, the properties of active metabolites, half-life and patient characteristics that would decide which drug is prescribed (Maxwell, 2012).

Phenazepam (**16**) (7-Bromo-5-(2-chlorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one) (Figure 2.6) with a potency around five times that of diazepam (**1**), was developed in Russia and although not prescribed in the UK, it is medically used in some other countries, where it is prescribed for anxiety and insomnia (World Health Organisation, 2015c). Phenazepam (**16**) use became more widespread through internet sales, leading to it becoming a controlled substance in the UK in 2012 (United Kingdom Government, 2012).

A new designer drug, pyrazolam (**17**), appeared on the market in 2012 under the description of ‘research chemical’, opening the door to a variety of substances that were not used medically anywhere in the world (Moosmann *et al.,* 2013). This includes flubromazepam (**8**) and diclazepam (**6**), which unlike phenazepam (**16**) are manufactured entirely for the recreational market. Flubromazepam (**8**) is similar in structure to phenazepam (**16**) with the bromine substituted for a fluorine atom (Figure 2.9). Diclazepam (**6**) is more structurally similar to diazepam (**1**), with an extra chloride (O'Connor, Torrance and McKeown, 2016) (Figure 2.9). Other

substances which were legally sold at the time include meclonazepam, nifoxipam, nitrazolam and the thienodiazepines such as deschloroetizolam and metizolam (Manchester *et al.,* 2018). The introduction of the Psychoactive Substances Act of 2016 brought an end to the legal sale of such substances by preventing the production, supply, import and export of any psychoactive substance. Interestingly, the potential for benzodiazepine abuse was realised soon after their introduction and thirty-five different benzodiazepine type drugs were controlled under the UN Convention on Psychotropic Substances 1971 (United Nations Office on Drugs and Crime, 1971).

In addition to the designer drugs, potent drug substances that were prescribed in other countries, such as etizolam (**7**), continued to enter the UK. Etizolam (Figure 2.9) is a thienotriazolodiazepine developed in Japan with a potency ten times higher than diazepam (**1**) and became a controlled substance listed in the Misuse of Drugs Act 1971 (Amendment Order) 2017 (Figure 2.9) (United Kingdom Government, 2017b; World Health Organisation, 2015a).

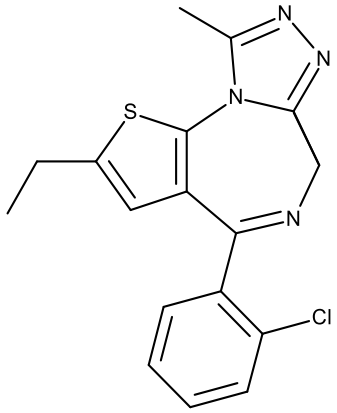
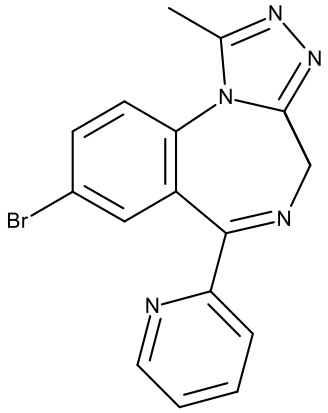
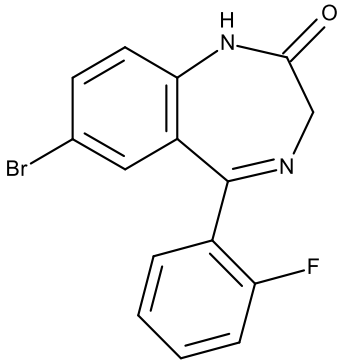
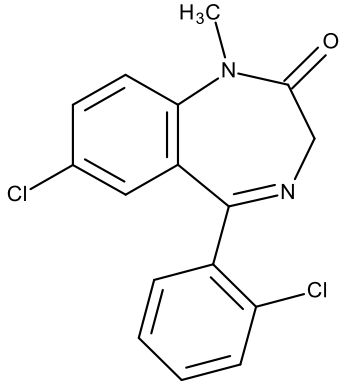
Etizolam	Pyrazolam
	
Flubromazepam	Diclazepam
	

Figure 2.9. Structure of four of the substances that were legally sold in the UK until the Psychoactive Substances Act of 2016 and the Misuse of Drugs Act 1971. (Amendment Order) 2017 (Home Office, 2016; United Kingdom Government, 2017b)

2.2.4 The Rise in Popularity of Diazepam

Due to the relative safety of the benzodiazepines compared to previously available anxiolytics, there was a 110% rise in benzodiazepine prescriptions between 1965-1970 and they became the most prescribed tablets across the world within ten years of their introduction (López-Muñoz, Álamo and García-García, 2011). By 1977, diazepam (**1**) was estimated as being taken by one in ten adults in the USA and it has been suggested that this was because of a rise in social stress leading to diazepam (**1**) being prescribed for non-medical problems (Waldron, 1977).

The benefit of diazepam (**1**) over other benzodiazepines is the speed of absorption and the duration of the drug substance, which limits potential fluctuations in blood concentration. Active substances with higher potency, such as alprazolam (**2**), are expelled more quickly, causing a drop in levels and leading to anxiety between doses. Less potent prescriptions such as chlordiazepoxide (**3**), with a maximum daily dose of 100 mg for anxiety, may not be the most suited for the symptoms presented (Ashton, 1994), though the dose of chlordiazepoxide (**3**) can be repeated after 2 hours in the treatment of severe alcohol withdrawal (Dr Reddy's Laboratories (UK) Ltd, 2015)

Data supplied by the National Health Service in Scotland (Figure 2.10) indicates that the number of diazepam (**1**) prescriptions had been on a steady rise over the last decade, reaching a peak of 893,146 in 2011/12 (Information Services Division, National Health Services Scotland, 2016). Over the last few years there has been a slight downward trend in the number of prescriptions, possibly due to concerns over tolerance and dependence that develop with long term usage (Listos, Talarek and Fidecka, 2010).

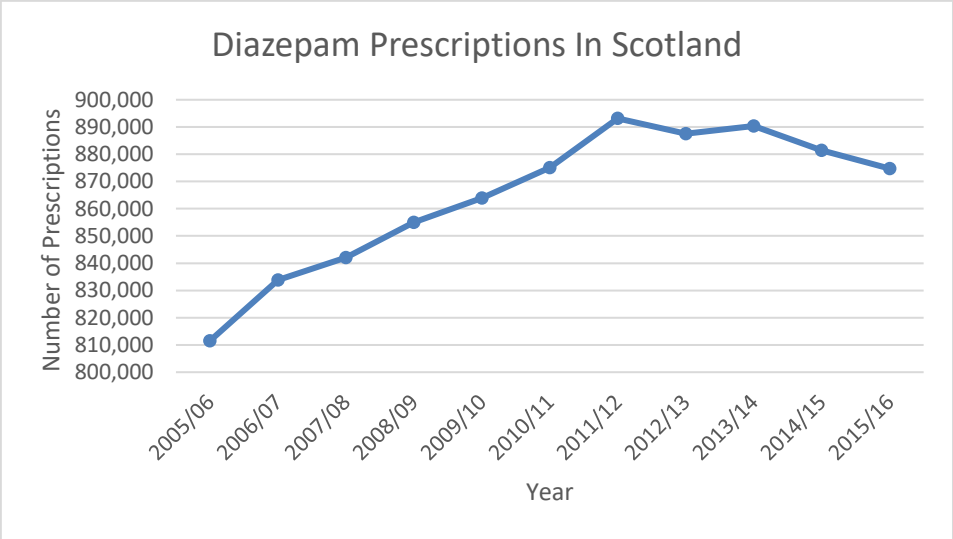


Figure 2.10. The number of diazepam (1) prescriptions dispensed in Scotland since 2005/6. (Information Services Division, National Health Services Scotland, 2016)

2.2.5 Illicit Benzodiazepine Use

Benzodiazepines were originally marketed as a safer option to barbiturates, with cases of benzodiazepine overdose leading to unconsciousness as opposed to the fatalities caused by high concentrations of barbiturate (López-Muñoz, Álamo and García-García, 2011; Cappell, 1986). However, there is a greater risk posed to those with a history of substance abuse, the elderly and patients with respiratory or renal disease or when ingested in conjunction with other active substances or alcohol (Palmaro, Dupouy and Lapeyre-Mestre, 2015). For example, the arrhythmic effects experienced by more vulnerable subjects when diazepam (**1**) is ingested at pharmaceutical levels, are heightened when the amount is increased. It has been reported that substance abusers often consume up to ten times the prescribed dose (Johnson, Barnsdale and McAuley, 2016).

Studies have indicated that a control group of subjects who have never used benzodiazepines, do not tend to favour benzodiazepines but they have more appeal to patients who had previously been prescribed for medical reasons and/or those with a history of drug or alcohol abuse (Cappell, 1986). Screening interviews of patients undergoing drug abuse treatment in America indicated that benzodiazepines

produce a mild feeling of euphoria and therefore they would preferentially choose more potent substances such as cocaine or MDMA. However, it was noted that benzodiazepines were taken to boost the effects of other active substances including methadone. Trials revealed that diazepam (**1**) was the most popular benzodiazepine and in high doses was sometimes believed to be a barbiturate (J. O. Cole and Chiarello, 1990). In 2016, records in the United Kingdom indicate that three times more people listed benzodiazepines as their secondary substance when referred to treatment centres, than their primary drug of abuse. Over two thirds of these cases identified heroin as the main active drug substance used (United Kingdom Government Focal Point on Drugs, 2017). However, it has been suggested that secondary drug substances are frequently under-reported (Johnson, Barnsdale and McAuley, 2016). Therefore, the rise of diazepam (**1**) abuse is not isolated but is reflected in the abuse of other depressant type substances.

Interestingly, in surveys, subjects taking part in trials and recognising the effects of diazepam (**1**), ascribed a higher monetary street value to diazepam (**1**) than other benzodiazepines, such as oxazepam (**15**) (Griffiths *et al.,* 1984), which introduces a motive for clandestine laboratories and illicit traders.

2.2.5.1 Polydrug Use

Benzodiazepines are commonly ingested at high levels, in conjunction with alcohol to speed up and intensify the intoxification; with cocaine to alleviate agitation and irritability; and opioids to enhance the euphoric effect (Stevens and King, 2013). Polydrug use exacerbates respiratory depression through synergistic effects, which is especially dangerous with opioids, antidepressants, and antipsychotics (Dear and Bateman, 2016). In addition, competition for metabolic pathways leads to an increase in drug concentration levels within the body and is exacerbated when the active substances are ingested in larger quantities (Johnson, Barnsdale and McAuley, 2016).

The combined use of benzodiazepines and opioids can trigger physical and psychological problems. Drug interactions can cause selective serotonin reuptake inhibitors to raise diazepam (**1**) levels in the blood and during metabolism,

interactions exacerbated by hepatic dysfunction can raise the concentration of opioid serum in the body, thus increasing sedation and the likelihood of overdose (Stevens and King,2013; Stein *et al.,* 2017; Johnson, Barnsdale and McAuley, 2016). A patient survey performed in a drug treatment centre in America, revealed that almost a third of the subjects admitted to having overdosed during the previous year. However, their continued use of benzodiazepines indicates a potential dependency (Stein *et al.,* 2017). In addition, some overlap exists in the withdrawal symptoms of benzodiazepines and opioids, emphasising the potential connection in neurochemical pathways. Benzodiazepines influence the impact of the GABA system on the locus coeruleus, aggravating opiate withdrawal symptoms (de Wet *et al.,* 2004).

2.2.5.2 Drug Related Deaths

Dangers posed by drug addiction and possible overdose are dependent on the types of drugs consumed. However, the problem is heightened by polydrug use and one of the most common drugs used in conjunction with other drugs and/or alcohol is diazepam (**1**), which is often taken to enhance euphoric effects, or for the alleviation of agitation and anxiety (J. O. Cole and Chiarello, 1990). This polydrug use is dangerous and has the potential of resulting in coma or death. In 2002, it was noted that the majority of people who died in amphetamine, cocaine or MDMA related incidents were also found to have consumed benzodiazepines and / or opiates, usually in conjunction with alcohol. It was even recorded that the most common combination was morphine, alcohol and benzodiazepines (EMCDDA.Europa.EU, 2002).

Problems related to polydrug use have been highlighted by the National Institute on Drug Abuse in the USA. Data released in 2017 year reveals a dramatic difference in the number of deaths related to benzodiazepines when taken with opioids, compared to benzodiazepines alone. The figures show that between 2002 - 2015, benzodiazepine deaths involving opioids increased twice as much as those that did not involve opioids (Figure 2.11).

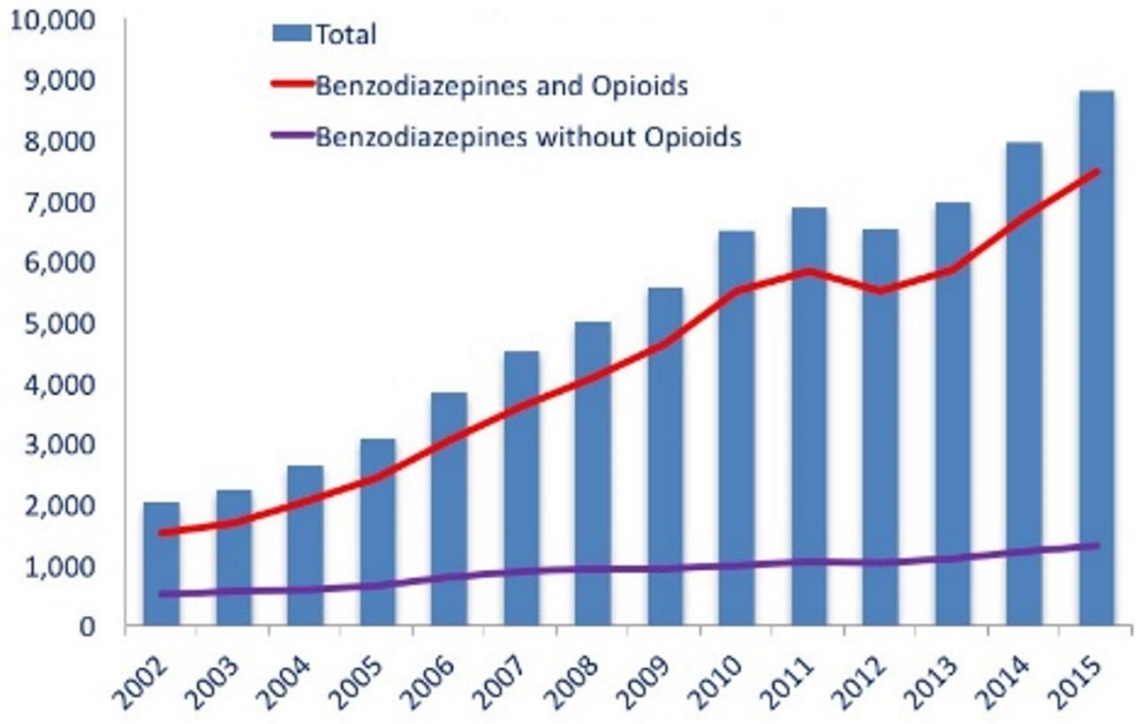


Figure 2.11. Comparison of benzodiazepine related deaths in the USA, when taken with and without opioids. (National Institute on Drug Abuse, 2017)

Records in England and Wales during 2016, indicated that the number of drug related deaths had increased by 2% over the previous year, reaching a record high of 3,744. Around 69% of the drug related deaths were listed as being the result of drug misuse. The number of deaths identifying specific substances indicate that benzodiazepines were implicated in 406 deaths during 2016. This is a rise of 10.9% over the previous year (Office for National Statistics, 2017). Proportionately the number of deaths involving benzodiazepines is much lower in England and Wales than recorded in Scotland.

In Scotland, there were 867 drug-related deaths recorded during 2016, an increase of 23% compared to 2015. Of these, benzodiazepines "were implicated in, or potentially contributed to" 49% (426 deaths) (National Records of Scotland, 2017). In 2015, benzodiazepines were implicated in only 27% (191) of drug related deaths, indicating that there was a massive increase of 123% over the previous year.

The statistical reports on drug related deaths in England and Wales, as well as the report for Scotland, both use records of all registered deaths in conjunction with International Statistical Classification of Diseases and Related Health Problems (ICD) codes. The ICD codes identify the cause of death based on the registration of death certificates. In Scotland, further information is gained from certifying doctors, pathologists and Procurators Fiscal. This information is obtained through a questionnaire, which requests details of any drugs or solvents found. The questionnaire was updated in 2014, allowing greater detail and differentiation between the identified substances (Office for National Statistics, 2017; National Records of Scotland, 2017).

A comparison showing the numbers of drug related deaths with those linked to benzodiazepines can be seen in Figure 2.12. The report on drug related deaths in Scotland released in 2017, indicates that the majority of cases involved the consumption of more than one active drug substance, thus emphasising the dangers posed by polydrug use (National Records of Scotland, 2017).

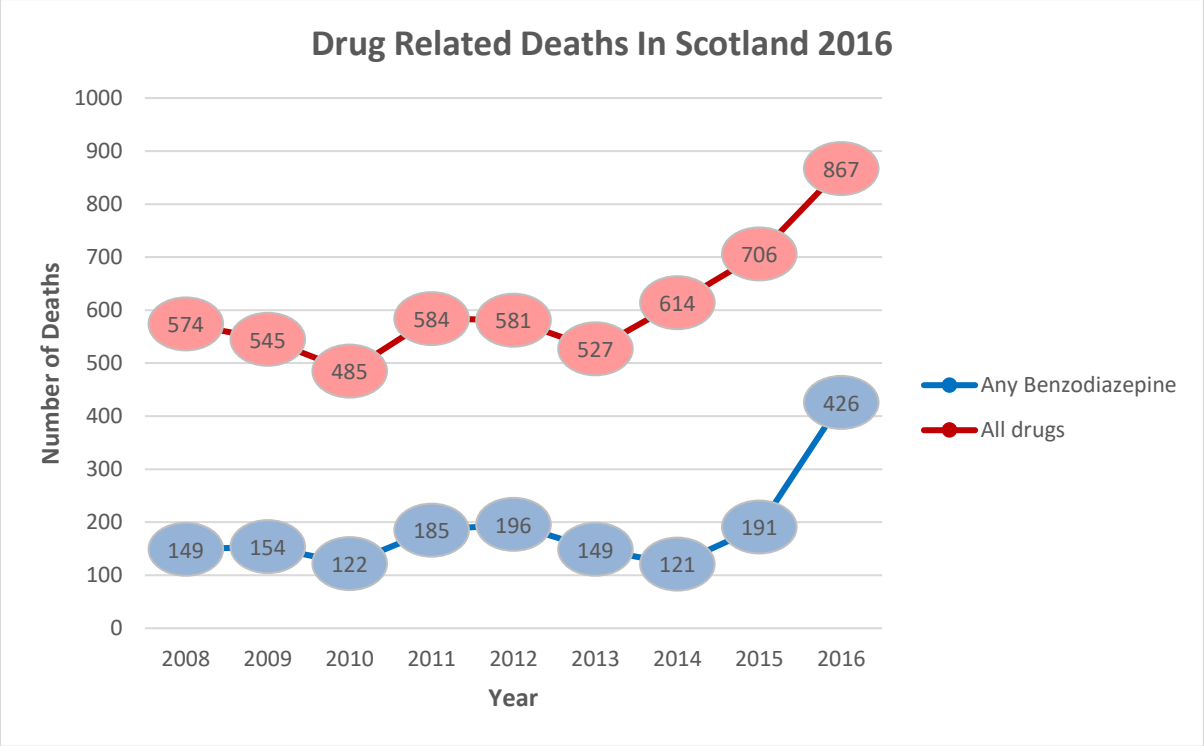


Figure 2.12. Comparison of the number of drug related deaths in Scotland, to those where benzodiazepines were identified. (National Records of Scotland, 2017)

According to the National Records of Scotland report on drug related deaths during 2016, benzodiazepines were identified in 277 out of 286 incidents, where New Psychoactive Substances (NPS) had potentially contributed to or been implicated in a death. Although a variety of substances were identified, the most common NPS found was etizolam (**7**). The number of incidents relating to etizolam (**7**) and diclazepam (**6**) rose at a much faster rate than for diazepam (**1**) (Figure 2.13). Until recently, diazepam (**1**) had been the most commonly identified benzodiazepine in drug related deaths (National Records of Scotland, 2017). However, whether the consumers had chosen to change active ingredients or were taking tablets unwittingly believing them to contain diazepam (**1**), cannot be determined. Potentially, illicit manufacturers could have swapped drug substance in order to stay within the law because etizolam (**7**) for example, was not controlled until the Psychoactive Substances Act of May 2016. However, it is now controlled as a class C drug under the Misuse of Drugs Act of 1971, due to an amendment in May 2017 and has been listed as Schedule 1 (United Kingdom Government, 2017b). As described in section '2.1.3 – Benzodiazepine Family', diclazepam (6), which was recorded as being on the rise, has no medical use but was produced exclusively for the recreational market.

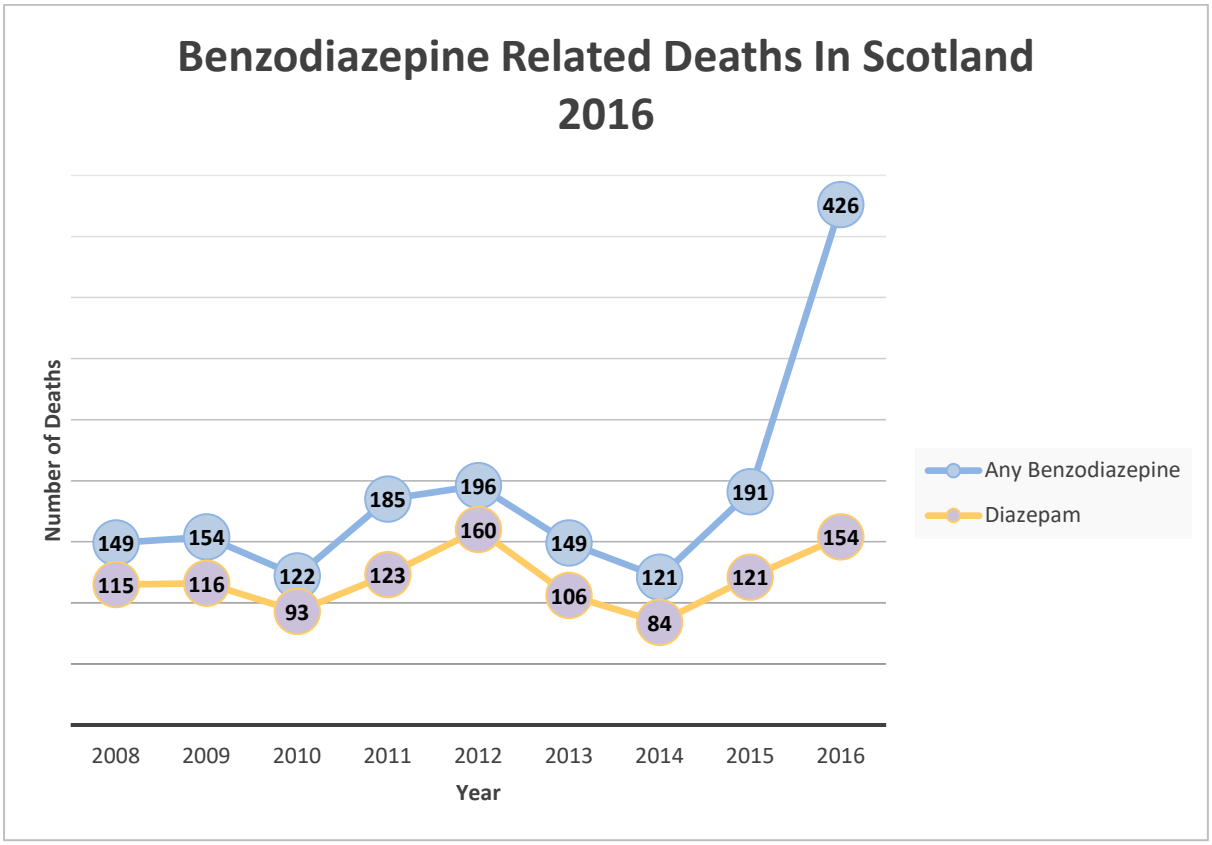


Figure 2.13. Comparison of the total number of benzodiazepine related deaths in Scotland, to those where diazepam (1) was identified, since 2008. (National Records of Scotland, 2017)

Although the number of drug related deaths in Scotland has been reported as being the highest in the European Union, differences in reporting and areas covered make true comparison difficult (National Records of Scotland, 2017).

2.3 Pharmaceutics

2.3.1 Formulation of Tablets

Tablet formulation is vitally important in the manufacture of pharmaceutical products. Ethical pharmaceutical tablets are designed to ensure a consistent medicinal dosage is provided, while ensuring stability and the correct bioavailability over the term of their shelf-life (Narang, Desai and Badawy, 2012). The bioavailability is the level of the administered drug substance that enters the systemic circulation enabling it to have an effect (Aulton, 2007b). However, there are many influencing factors, including particle size, dissolution properties, ease of handling, and the flow and compression properties. This means that a variety of constituents (excipients) have to be added along with the drug substance, to ensure the viability of the tablet. In addition, interactions between the different substances have to be monitored to safeguard the integrity of the product.

Formulations vary between manufacturing companies and therefore not all the excipients will be the same or create the same interactions within a tablet. However, the final product is registered with the Medicines and Healthcare products Regulatory Agency (MHRA), who monitor the quality and specifications of each product.

2.3.2 Impact of Particle Size

Particle size has a significant impact on tablets and their properties as it affects content uniformity, particularly in low-dosage tablets. Pharmaceutical blue diazepam (**1**) tablets produced for the UK market and analysed for this project (chapter 3 – Physical Characteristics), ranged in weight between approximately 148 – 180 mg, of which 10 mg is diazepam (**1**) and the remaining weight relates to a variety of excipients. By incorporating the active ingredient as a larger quantity of smaller particles, a more consistent batch can be produced. Small particles also provide a greater surface area to maximise dissolution and absorption rates (Fu *et al.,* 2012; Aulton, 2007b).

2.3.3 Crystal Properties of Molecules

Solid components used to manufacture tablets exist in crystalline, amorphous and polymorphic forms. Loosely packed molecules are more likely to fragment, dissolve or melt, which impacts on tablet compaction, storage and use. Although some substances are enantiotropic, meaning they can change between different equally stable polymorphic forms under certain conditions, many are not. Substances used in pharmaceutical formulations are often monotropic, meaning that one of the polymorphs is more stable and the less stable forms will convert over time. Consequently, the crystalline structure of some tablets changes over time, reducing the bioavailability of the active ingredient but the rate of conversion fluctuates according to storage conditions. (Aulton, 2007b). In order to limit these problems, analytical tests are often performed to check for the presence of polymorphic forms (European Medicines Agency, 2000).

The level of amorphous content also influences the crystal state and is affected by the glass transition temperature. Samples kept below this temperature will be hard and brittle with no mobility. However, above the glass transition temperature the molecules become more fluid, enabling them to recrystallize in a more regular structure.

The absorption of moisture by amorphous substances can act as an effective plasticizer by lowering the glass transition temperature. The added water can either form hydrogen bonds with the polar molecules in the structure, which binds the water within the network; or if the substance is more hydrophobic, the molecules will create distance by breaking the secondary bonds that already exist within the molecule. Both of these effects allow movement of the molecules, enabling them to find a more stable form (Smith *et al.,* 2009). Unbound water content may be expelled through the crystallisation process. However, some substances including lactose, which is a common tablet excipient, retain moisture and are converted into a monohydrate (Aulton, 2007b).

2.3.4 Tablet Excipients

The term ‘excipient’ relates to all the constituents added to a tablet, other than the active drug substance. Excipients are “inert substances” with no biological effects however, they significantly influence the absorption rates of active drug substances (Aulton, 2007b). Each excipient has its own function and some compounds have multiple roles. The purpose of many of the excipients is described below. Formulations vary between different manufacturing companies but each factor requires consideration and is tested to assess the interactions taking place between the various constituents.

2.3.4.1 Diluents Used In Tablet Manufacture

The use of a diluent or bulking agent is important for giving volume to tablets containing a small amount of active drug substance. Bulk is required for tablet compression and manageability (Lachman, Lieberman and Kanig, 1970). A tablet containing only 10 mg of diazepam (**1**) on its own would not be practical. Ideal diluents should be biocompatible, chemically inert and non-hygroscopic with good solubility and compaction properties (Aulton, 2007b). The most common bulking agent used in diazepam (**1**) tablets produced for the UK market is lactose (Medicines and Healthcare Products Regulatory Agency, 2014; Medicines and Healthcare Products Regulatory Agency, 2016b; Medicines and Healthcare Products Regulatory Agency, 2015b; Medicines and Healthcare Products Regulatory Agency, 2015c; Actavis UK Ltd, 2014)

Lactose exists as both crystalline and amorphous structures. Either the α-monohydrate or the anhydrous β-lactose are produced depending on the crystallisation conditions. Amorphous lactose is easier to dissolve and compact but is hygroscopic and unstable. High relative humidity and a rise in temperature can cause crystallisation (Aulton, 2007b).

2.3.4.2 Disintegrants Used In Tablet Manufacture

Disintegrants are added to the formula in order to facilitate the breakdown of the tablet thus creating a larger surface area for dissolution. Disintegrants work in two ways: firstly, some disintegrants promote the uptake of water by increasing hydrophilicity and the interfacial tension is reduced and the tablet disintegrates when it becomes wet. Secondly, the disintegrant particles swell during sorption of water, causing the tablet to rupture. The most prevalent disintegrant used in tableting is starch, which works by the latter method (Aulton, 2007b). Maize starch is commonly used in the diazepam (**1**) pharmaceutical tablets produced for the UK market (Medicines and Healthcare Products Regulatory Agency, 2014; Medicines and Healthcare Products Regulatory Agency, 2016b; Medicines and Healthcare Products Regulatory Agency, 2015b; Medicines and Healthcare Products Regulatory Agency, 2015c; Actavis UK Ltd, 2014)

2.3.4.3 Binders Used In Tablet Manufacture

The use of binders helps to hold a tablet together and gives the required mechanical strength. Binders can be added as a dry powder or as a solution depending on the tableting method. The binder used for diazepam (**1**) tablets by MA Pharmachem and Wockhardt, is polyvinyl pyrrolidone (PVP), otherwise known as Povidone (Medicines and Healthcare Products Regulatory Agency, 2015c; MA Pharmachem Ltd, 2011). Povidone is a synthetic polymer, which has increased adhesive properties. Due to its hydrophilic nature, Povidone can also act as a disintegrant and can reduce the speed of polymorphic conversion of crystals by working as a viscosity-inducing agent (Panakanti and Narang, 2012).

2.3.4.4 Glidants Used In Tablet Manufacture

Glidants are used to improve the smooth flowing of powders through the tableting machine. This restricts blockages and helps to promote correct filling of the dies. Ensuring consistency within tablets and between batches in the pharmaceutical industry is of the utmost importance. Consistency is affected by particle shape and size, the effectiveness of the mix and the ease of flow through the tableting machine. Irregular flow leads to uneven filling of the die wells on the tableting machine,

potentially producing fragile tablets due to incorrect compression, which, could result in tablets containing an incorrect blend and uneven dosage.

Spherical particles flow more easily than irregular crystals, which are more likely to interlock. This results in the rough crystals clumping together creating an uneven, more heterogeneous blend. There are a variety of measures which can be taken to limit these problems. For example, certain additives can be introduced which are designed to encourage crystal growth on particular faces during the production process. In terms of tableting, glidants such as talc, colloidal silicon dioxide or magnesium stearate can be added to help reduce the bulk density. Diazepam (**1**) tablets in the UK contain magnesium stearate, which has a dual purpose as both a glidant and a lubricant (Medicines and Healthcare Products Regulatory Agency, 2014; Medicines and Healthcare Products Regulatory Agency, 2016b; Medicines and Healthcare Products Regulatory Agency, 2015b; Medicines and Healthcare Products Regulatory Agency, 2015c; Actavis UK Ltd, 2014; Aulton, 2007b).

2.3.4.5 Lubricants Used In Tablet Manufacture

Lubricants are used to enable compressed tablets to slide out of the die wells (Lachman, Lieberman and Kanig, 1970). They are generally fine powders which create a thin barrier between the tablet and die to reduce friction.

However, there are a number of problems associated with lubricants. For example, agglomeration of particles can increase through static charge of the fine powder. Also, as lubricants are often lipophilic, they can slow down the dissolution rate of the tablet by increasing hydrophobicity. Magnesium stearate used in the manufacture of some pharmaceutical diazepam (**1**) tablets (MA Pharmachem Ltd, 2011), is hydrophobic and although it is useful as both a lubricant and anti-adherent, it can hinder the dissolution of tablets by adhering to the surface of the tablet creating a film (Ariyasu, Hattori and Otsuka, 2016). Quantities of magnesium stearate are therefore kept to a minimum. Alternatively, a water-soluble surfactant and hydrophilic diluent can be added. Surfactants can aid dissolution by decreasing the interfacial tension between solid and liquids thus allowing the solid particles to become wet. They also have the ability to disrupt membranes in the gastro-intestinal

tract, increasing absorption. This requires careful monitoring however, as an increase in absorption and bioavailability could have potentially toxic consequences (Aulton, 2007b).

The choice of excipient is important because some substances interact to the detriment of bioavailability or by increasing toxicity. The presence of magnesium stearate for example, can increase the alkalinity and encourage hydrolysis of some active ingredients. The effects may be further influenced by the presence of magnesium oxide which is an impurity produced during the manufacture of magnesium stearate, thus increasing the level of alkalinity. Alternatively, stearic acid being used as the lubricant can help to protect some active drug substances from degradation. Further interactions with magnesium stearate can occur through oxidation, interactions with primary amines and the magnesium metal ions leading to metal chelation (Li and Wu, 2014). The pharmaceutical company Actavis list both magnesium stearate and stearic acid in their list of ingredients in their 10 mg diazepam (**1**) tablets (Actavis UK Ltd, 2014).

2.3.4.6 Anti-adherents Used In Tablet Manufacture

Anti-adherents, including magnesium stearate, prevent tablets from sticking to the punch or die wall (Lachman, Lieberman and Kanig, 1970). Sticking is related to the moisture content of the tablet and is a common problem, which is particularly prevalent with patterned punches. Accumulation of powder on the dies can create uneven tablets with indistinct logos.

The tableting of illicit preparations can be performed in a variety of ways. Firstly, a paste can be made containing the active ingredient, excipients and dyes, which are placed into a mould and allowed to dry. This forms a more rough and uneven tablet, without logo and bearing little resemblance to pharmaceutical tablets and therefore creating tablets less affected by the anti-adherent. This is an older method that is rarely used industrially but may still be used in some illicit circles.

A much more common process is the use of tablet presses (Humphries, 1984). The use of a single punch press means that dies containing pharmaceutical logos can allow distinguishing between illicit and legitimate tablets more difficult, especially as

the clandestine manufacturers perfect their formula to prevent sticking. However, incorrect alignment or mishandling of the equipment can lead to punch marks which can link tablets back to the press. The more sophisticated multi-station press makes the linking of tablets less likely as many more tablets are manufactured at the same time and not necessarily bearing the same logo (Humphries, 1984).

2.3.4.7 Colourants Added in Tablet Manufacture

Colourants are added to tablets for a number of reasons, including as a complement to the flavour, to mask a less appealing colour or to hide a colour change. For example, primary amines can interact with amorphous lactose in a Maillard Reaction, resulting in a brown discolouration of tablets (Rowe, Sheskey and Weller, 2003). This reaction has also been reported with secondary amines but not with nitrogen present in heterocyclic rings (Narang, Desai and Badawy, 2012).

Dyes may be added as a coating or as an integral part of the tablet but blue diazepam (**1**) tablets produced for the UK market have the colourant blended throughout. Colour also enables standardisation and helps with tablet identification. The colourant helps to identify the strength of the different tablets, so for diazepam (**1**) produced for the UK market, 10 mg tablets are blue, 5 mg yellow and 2 mg are white. This colour coding therefore assists in tablet differentiation and housekeeping for pharmacists, but although all 10 mg diazepam (**1**) tablets from UK manufacturers are blue, the type of dye used may vary.

The dyes used can be oil or water soluble but may also be in lake form. Aluminium lake complexes are insoluble in water or oil and demonstrate an increased stability to light and pH compared to the dye alone (Aulton, 2007b). In the UK, the most common dye used in 10 mg pharmaceutical diazepam (**1**) tablets is indigo carmine (E132). However, Actavis list the indigo carmine lake as their colourant (Actavis UK Ltd, 2014) and Bristol laboratories use dispersed blue 12726 (Medicines and Healthcare Products Regulatory Agency, 2015a), which is an alumina lake (Harvey and Hayes, 1984).

2.3.4.8 Pharmaceutical Excipients

Each company produces an information leaflet to inform consumers of the constituents of their tablets. MA Pharmachem for example, list their ingredients as: lactose for the diluent, making up the majority of the bulk; maize starch is used as the disintegrant, Povidone or PVP as the binder and magnesium stearate as the glidant, lubricant and anti-adherent. The colourant used by MA Pharmachem is indigo carmine dye (E132).

2.3.4.9 Interactions between Excipients

Excipients are “inert substances” but they do interact with each other and the active ingredients, impacting on the bioavailability of the active drug substance. Excipients are intended to help the absorption process by ensuring the active drug substance is dissolved so that it rapidly reaches its absorption site. However, poorly chosen excipients could form complexes that have lower solubility or increased molecular size rendering them incapable of diffusing through cell membranes. Extensive tests are performed by the manufacturing companies to ensure interactions are beneficial and do not hinder the bioavailability (Panakanti and Narang, 2012).

In terms of diazepam (**1**) tablets, an investigation into incompatibilities between the active ingredient and excipients has been performed by Matos *et al.* (2017) This research entailed using Differential Scanning Calorimetry (DSC) and thermogravimetric analysis (TA), in conjunction with infrared spectroscopy (IR). The excipients tested were lactose monohydrate (diluent), microcrystalline cellulose (emulsifier and bulking agent), pregelatinized starch (binder), sodium starch glycoloate (disintegrant and gelling agent), croscarmellose sodium (super disintegrant), colloidal silicon dioxide (glidant, adsorbent, disintegrant and anti-caking agent) and magnesium stearate (lubricant). The study showed that an interaction took place between the diazepam (**1**) and colloidal silicon dioxide, however this was not significant enough to cause incompatibility. This interaction was detected using DSC but not by TA or IR and it was suggested that DSC was more sensitive for this type of analysis (Matos *et al.,* 2017).

2.3.5 Tableting Methods

There are two methods used for tablet manufacture by the pharmaceutical industry, granulation, which binds powders together to form granules before compaction; or direct compression, which uses pressure to force the powder to stick together as a cohesive unit (Aulton, 2007b). Granulation is the method used by MA Pharmachem (Wesley, 2014).

2.3.5.1 Granulation Method

The aim of granulation is to promote the even distribution of active drug substance and excipient content within pharmaceutical tablets. Granulation is an intermediary step which is usually performed after all of the tablet constituents have been mixed together, whereby the mixed powders adhere together to form granules, usually of around 0.2 - 0.5mm. Once the granulation has taken place, further excipients may be added before the tablets are compacted. Excipients added prior to granulation include diluents for bulking, disintegrants for granule fragmentation and adhesives that are predominantly added as a dry powder for this process. Lubricants are added after granulation and sometimes additional disintegrant or all of the disintegrant can be incorporated at this time (Aulton, 2007b).

Granulation is advantageous in pharmaceutical manufacturing for a variety of reasons. Firstly, as a mixture of components are combined in the granule, they are kept together during the tableting process (Aulton, 2007b). Through movement, powders segregate according to size and density, with the small and more dense particles accumulating at the bottom. Granules therefore help to maintain a uniform blend of constituents throughout a batch, providing a more uniform drug dosage.

Granules help to improve the flow of the blend as they tend to be more spherical in shape than powders (Figure 2.14). The flow is also aided by the larger size of the granules because they are often more able to absorb moisture in the system without affecting the flow. Powders which are more hygroscopic and those with an irregular shape and increased friction, can flow more slowly. Compaction is improved in granular methods, due to even dispersion of the adhesive within the structure, which results in more cohesive tablets. Compactability relates to the ability to form a robust

and viable tablet, which is resilient to fractures, capping and lamination (Aulton, 2007b).

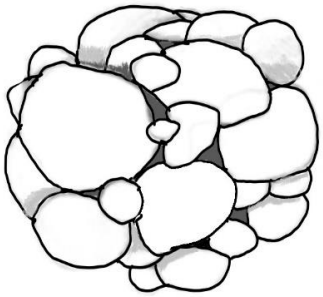


Figure 2.14. Sketch of a granule, showing a variety of components of different sizes combined into a coherent unit.

Granulation can be performed using either a dry or a wet granulation process. Dry granulation is preferred for more hygroscopic substances. The dry granulation technique uses pressure to aggregate the powder and this can be done in a heavy duty press to produce slugs, or by using two rollers resulting in a sheet. The products are then granulated by milling and granules of the required size are separated through sieves (Aulton, 2007b).

Wet granulation combines the powders with a granulating fluid, causing them to bind together. The fluid contains a volatile, non-toxic solvent such as water, ethanol or isopropanol, which is removed in a drying step. The solvent can be combined with binding agents such as Povidone, which maintain adhesion after drying. Wet granulation can either be performed on a fluidized-bed, where the powder is 'fluidized' by being blown with a stream of heated air, while being sprayed with the granulating fluid and then dried; or in a high-speed mixer. The high-speed mixer technique agitates the particles while fluid is progressively added to give an even coating, which helps to form and shape spherical granules (Saleh, Vialatte and Guigon, 2005). Low-shear wet granulation and high-shear wet granulation techniques both use a high-speed mixer but the low-shear method uses a separate granulator, whereas it is an integral part of the high-shear system.

The method of granulation used impacts on the pore structure both within and between granules by influencing the level of packing between the granules, for example, granules produced by the fluid-bed are not as tightly packed as those that had been sprayed (Aulton, 2007b). The inclusion of an adhesive in wet granulation techniques increases the strength of the tablets. A study comparing granulation by fluidized-bed to low and high-shear methods, to create a low dose soluble active tablet was performed by researchers at Procter & Gamble (Hausman, 2004). The experiment combined an unnamed active drug substance with lactose monohydrate, microcrystalline cellulose, Povidone, Crospovidone (polyvinyl polypyrrolidone, which is a cross-linked version of povidone) and magnesium stearate. Many of the excipients used in the experiment are formulated into pharmaceutical diazepam (**1**) tablets in the UK. The test concluded that in terms of producing a viable cohesive product, the fluidized-bed method was effective as low and high-shear granulation methods.

2.3.5.2 Direct Compression Method

In direct compression the active drug substance and excipients are mixed and tableted. Although this can prove a speedier and less expensive option, the specially formulated binders and other excipients can increase the cost. Disadvantages include the increased likelihood of particle segregation and a reduced level of compactability (Aulton, 2007b).

Direct compression is preferred for soluble or potent drug substances, where they can be combined with course excipient particles, to improve flow and even distribution of active drug substance. Other benefits of direct compression include an improvement in tablet stability because water and heat are not required in the manufacturing process and disintegration rates can increase due to particles being held together with weaker bonds. Dry granulation and direct compression use Van der Waals forces which increase in magnitude as the particles are forced together at pressure.

In contrast, wet granulation distributes a liquid film around the particles, which forms liquid bridges. As the granule dries, the added adhesive hardens creating a solid

bridge between the particles. Adhesives which bind in this way include PVP, which is present in the 10 mg diazepam (**1**) tablets produced by Wockhardt. Partial dissolution of powdered components through wet granulation, can result in crystallisation binding the particles as they dry. Lactose crystallises in this way, using water as the solvent in the wet granulation process. Bonds created during crystallisation produce stronger, more robust tablets (Aulton, 2007b).

Clandestine laboratories tend to use the direct compaction method of tableting, by compressing the free flowing powders (Humphries, 1984) which avoids the added expense of purchasing granulation equipment. However, research into the manufacture of ecstasy tablets revealed that another method of tableting involved a combination of the moulded method of dye filling, whereby the mixture is fed into a moulded plate, pressed with a steel punch to even it and then compressed by hitting it with a hammer (Baer, 2007).

2.3.6 Tablet Manufacture

The blend of granules and/or powders is poured into the tableting machine through a hopper, or funnel. From there it flows by gravitational force towards the die table and into the die. The die is a well in the die table, which has the lower punch positioned at the bottom. The location of the lower punch determines the amount of powder that will be compressed into the tablet, thus affecting tablet depth. The lower punch may be stationary or could move upwards during compression.

Once the die has been filled, the upper punch enters the die well from above and compresses the powder into a tablet. The upper punch withdraws and the lower punch moves up the die, allowing the tablet to be ejected.

2.3.6.1 Tablet Presses

Two main types of press are used for manufacturing tablets: - the single-punch press or the rotary press. Hydraulic presses are often used for development stages or research but are not usual for product manufacture (Aulton, 2007b).

A single-punch press can produce around 200 tablets per minute and is mainly used for small batches of tablets, such as in clinical trials. Single- punch presses have

one die and set of punches (Figure 2.15). The powder flows from the hopper into a hopper shoe above the die table. The hopper shoe moves across until positioned over the die, allowing the powder to fall in to the well and then returns to allow the upper punch to descend. The lower punch in a single-punch press is stationary and does not push upwards during compression. The pressure is therefore applied from above by the upper punch. The new tablet is then ejected and pushed away by the hopper shoe as it returns to refill the die well (Aulton, 2007b).

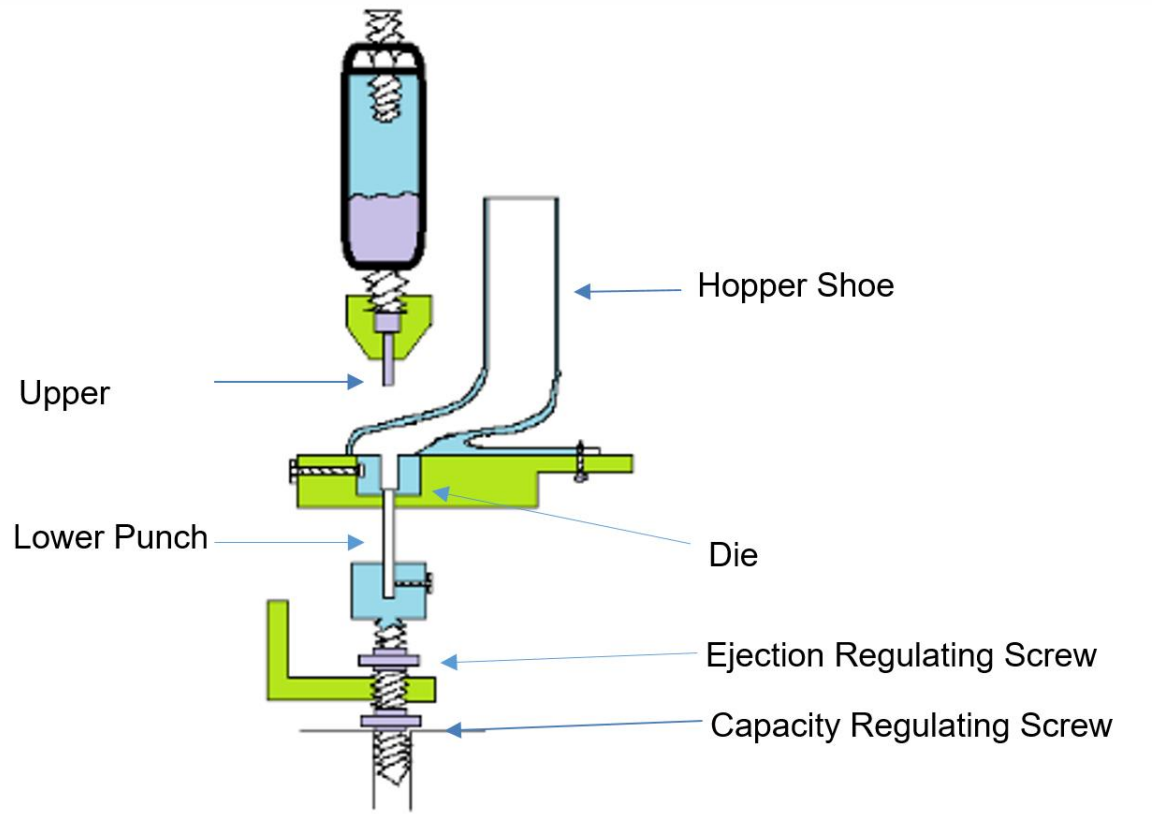


Figure 2.15. Diagram of a single- punch tablet press. (Aulton, 2007b)

The rotary press is a multi-station press, which can produce over 10,000 tablets per minute. The number of die and punch sets can vary from three to over 60 for large scale operations. The dies are positioned in a circle around the die table and the table rotates with the punches so that the same punch is always used for each die (Figure 2.16). Compression is completed by the vertical motion of both the upper and lower die.

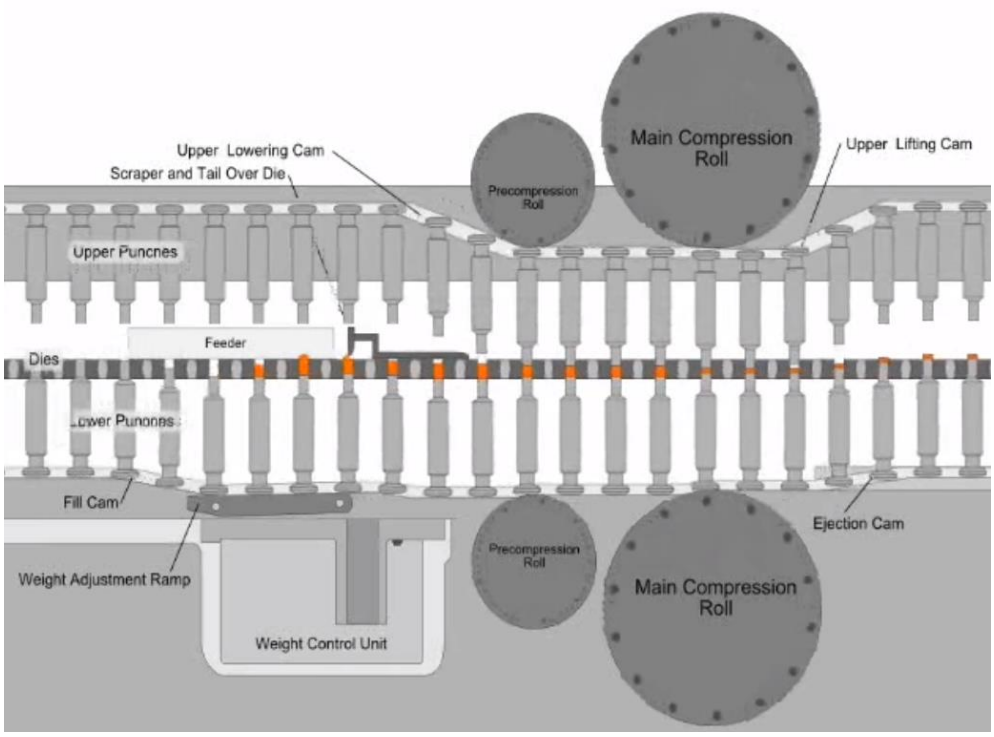


Figure 2.16. Diagram of rotary press mechanism. (Sajib, 2011)

Machine maintenance and cleaning to keep the surfaces smooth helps to limit technical problems. Most tableting problems result from powder content, such as variability in dosage or weight, uneven flow, sticking to the punch, low mechanical strength and capping or laminating. Capping is where the top of the tablet separates from the bulk of the tablet and lamination is a horizontal split across the tablet.

The availability of tableting machines on the internet allows clandestine manufacturers easy access to more sophisticated equipment. Research into ecstasy tablets noted that although older or second hand machines may be used by illicit manufacturers, the investigation had identified that newer and more powerful tableting machines were also used in clandestine laboratories (Baer, 2007). This can provide a more professional appearance for the illicit tablets and allows them to be produced in greater numbers.

2.3.6.2 Compression of Powders

Compression of powder takes place over several stages:

1. Rearrangement of the powder within the die.
2. Once a certain pressure is exerted, the particles no longer move due to the finite space and an increase in inter-particulate friction.
3. Particles undergo elastic deformation, plastic deformation or brittle deformation. Viscoelastic recovery means that powders that undergo elastic deformation can revert on decompression, or may reverse more slowly. Plastic deformation is a permanent alteration and brittle deformation results in fragmentation into smaller particles. The type and level of deformation is dependent on the formulated powders and the pressure exerted (Roopwani and Buckner, 2011).
4. The smaller particles then repeat the compression process beginning with rearrangement, as further force is exerted.
5. As the particles are forced together, agglomerations form.
6. On decompression, powders which underwent elastic deformation begin to revert back, breaking some of the newly formed bonds (Buckner, Friedman and Wurster, 2010).

The stages were confirmed in a study by Roopwani, who investigated the compaction behaviour of some common pharmaceutical excipients, including lactose, varying grades of corn starch and microcrystalline cellulose. It was found that at lower pressure, there was rearrangement of the powder and some fragmentation. Plastic deformation increased with the pressure, until available pore space was filled leaving nowhere for the particles to flow. At higher pressure, there was an increase in elastic deformation. These results were supported by the statistical principal component analysis used to examine the generated data. The statistical analysis also identified differences in the change of decompression density of pre-gelatinized corn starch, which was believed to relate to its unique viscoelastic properties (Roopwani and Buckner, 2011).

Compression of granules is different to powder compression and can result in deformation of the granules and/or the powders within the granules. At low pressure,

granules rearrange within the die but due to the course nature of granules and better flow properties, this stage is less significant than for powders. As the pressure increases, granules undergo elastic and plastic deformation. In addition, intragranular porosity reduces, resulting in an increased density of the granules. Limited fragmentation does occur but to a lesser extent than deformation. Minimal attrition can also occur through friction with the die walls (Aulton, 2007b).

2.3.7 Tablet Properties

Properties of pharmaceutical tablets produced for the UK market are specified in the British and European Pharmacopeia, the MHRA and by the individual manufacturing companies. The manufactured tablets are required to meet a variety of quality control measures, which include:

1. Correct dosage and uniformity of dosage. Uniformity of the dose is tested by analysing a sample of tablets and calculating whether their contents lie within the stipulated limit of standard deviation from the mean.
2. Consistency of tablet appearance, weight and size. Uniformity of weight is batch tested by weighing a sample of tablets and calculating the mean.
3. Consistent release and bioavailability of active drug content.
4. Safety of content, with no harmful substance or microorganisms present
5. Mechanical strength to ensure the tablet will remain intact under reasonable conditions, for the duration of its lifetime.
6. Crystalline stability for the duration of its lifetime so that changes in amorphous content do not affect bioavailability of the active drug substance.
7. Appropriate packaging to ensure the integrity of the tablets. (Aulton, 2007b)

A study was performed into the relationship between mechanical strength and dissolution rates of diazepam (**1**) tablets. The experiment compared varying quantities of excipients including microcrystalline cellulose, lactose and magnesium stearate, with different mixing times and compression pressures. Results indicated that although the mechanical strength of the tablets was affected, this did not directly correlate with the dissolution rates of the tablets (Pesonen *et al.,* 1997).

A study using a computer-generated hologram, investigated the homogeneity of the surface of tablets containing the common pharmaceutical excipient, *Emcompress*™ (Dicalcium phosphate dihydrate) compared to those containing starch. The tablet surface is important because it impacts on dissolution, surface wetting and contact angles. The technique used the diffuse reflection of incident laser light from the surface of a tablet to create the hologram. The results indicated that the surface of the tablets containing starch were more homogenous than those containing *Emcompress*™. It was suggested that during the compression process starch was more likely to break and form new bonds than *Emcompress*™ (Ketolainen *et al.,* 1997).

Tablet manufacture is therefore a complex process. Some of the considerations are purely to help with the production of tablets, such as the inclusion of glidants, whereas others are concerned with bioavailability and safety of use. Illicit manufacturers may want to make tablets easily but are not necessarily as aware or even concerned about the impact of their formula. Therefore, irrespective of the active drug substance, the excipients used by illicit producers may cause harmful effects.

Little is known about excipients used in illicit diazepam (**1**) tablets but they are likely to differ between the illicit manufacturers based on their own experience and according to the substances that are readily available. It has been suggested that due to the complications posed by the tableting process, once an illicit manufacturer finds a formula that works, they are likely to stick to it (Baer, 2007).

In 2010, an investigation into excipients used as cutting agents in a variety of illicit preparations, performed by the Centre for Public Health, based at John Moores University, reported that amphetamine tablets were found to contain glucose, bicarbonate of soda and paracetamol. They also noted that previous interviews with drug dealers had revealed that they regularly added caffeine, ephedrine, paracetamol and sugars, although dimethyl sulphone had recently been found. This had also been discovered in methamphetamine tablets in Australia, along with caffeine and a variety of sugars. In addition, ecstasy tablets were reported to regularly contain lactose as the main sugar, accompanied by cellulose, calcium

phosphate, and calcium and magnesium stearate. However, these tablets were also found to contain additional active ingredients such as paracetamol, caffeine, ephedrine, procaine, amphetamine and 1- phenylethylamine. Interestingly the investigation also claimed that heroin samples were sometimes cut with diazepam (**1**) (C. Cole *et al.,* 2010).

The rise of internet sales has also impacted on excipient availability and the European Monitoring Centre for Drugs and Drug Addiction (2016) reported that not only could relatively cheap tableting equipment and die stamps be bought from China, the vendors could also provide pre-mixed coloured excipients, which therefore makes accessing the ingredients easier and negates the problems of finding a viable formula.

The differences between illicit cases of tablets therefore result from a number of factors from the formula and excipients used, to the tableting and compaction methods and the dies used. Each of the choices made by the illicit manufacturer impact on the final characteristics of the tablet and can provide useful information for linking together the illicit tablets. Therefore, this project aimed to investigate a variety of physical and chemical characteristics of the illicit tablets in order to detect any similarities that could identify links between the different cases. This was done by comparing the results of the different forms of analysis and the data obtained and by employing statistical clustering techniques and linear discriminant analysis to group cases with similar characteristics. This is a novel type of research that has never previously been performed on illicit blue tablets seized in Scotland which provides important intelligence for police.

2.4 Drugs and the Law

The growth of the opium trade in Asia alerted trading countries, particularly America, to problems created by drugs, which became recognised as an international problem. After the establishment of the League of Nations, drug production, levels and usage were monitored internationally. The list of controlled drugs increased, with the inclusion of cannabis by the 1925 Geneva Convention. However, the emergence of new drugs meant that further legislation was required and this was finally brought together in 1961, with the United Nations Single Convention on Narcotic Drugs. This described an order for drugs based on the level of perceived danger and the amount of restriction required. Concern over the increasing abuse of stimulants and hallucinogenic drug substances lead to The Convention on Psychotropic Substances, 1971. This legislation listed substances such as psilocybin and lysergide under schedule 1 and named diazepam (**1**) and other benzodiazepines in schedule 4 (United Nations Office on Drugs and Crime, 1971).

The Convention against Illicit Traffic in Narcotic Drugs and Psychotropic Substances, 1988 was implemented due to a rise in recreational drug use, which had sparked an increase in clandestine laboratories and international drug trafficking during the 1970s-80s. The legislation was aimed at targeting the drug traffickers and had provisions for money laundering, as well as restricting the availability of many drug precursors (United Nations Office on Drugs and Crime, 1971).

The international policies were embedded into drug legislation in each of the participating countries. In the United Kingdom, the Dangerous Drugs Act, 1951 supported the policies set out in the Geneva Convention and focussed on restriction of production, import and export of cannabis, opium and morphine (United Nations Office on Drugs and Crime, 1951). The Dangerous Drugs Act of 1964 addressed concerns about cannabis cultivation and use, while the Drugs (Prevention of Misuse) Act of the same year concentrated on criminalising the possession of amphetamines, which were believed to be related to juvenile crime (Reuter and Stevens, 2007). In 1965 a new Dangerous Drugs Act was drafted to consolidate the previous laws.

The 1968 Medicines Act, classified drugs into prescription only; (those which could only be sold by pharmacies) and medicines (which could be sold in any shop); and then further detailed in the Misuse of Drugs Act of 1971, which brought in the new classification system. The 1971 Act aimed to classify around 250 named drug substances along with salts, stereoisomers which have the same molecular structure but in a different spatial arrangement and other preparations, into three main classes, A, B and C, where substances which were believed to present the most potential danger, such as cocaine, heroin and morphine were classified as group A. Group B included substances such as amphetamine and codeine and, Group C, which were considered to pose a lesser threat, contained the benzodiazepines (United Kingdom Government, 1971). Offences and penalties related to each of the classification levels, were also listed and are shown in Table 2.1.

Table 2.1. The penalties for certain drug offences according to the Misuse of Drugs Act, 1971.

(United Kingdom Government, 1971)

Classification	Production or Supply	Possession
Class A	Life imprisonment, unlimited fine, or both.	7 years imprisonment, unlimited fine, or both.
Class B	14 years imprisonment, unlimited fine, or both.	5 years imprisonment, unlimited fine, or both.
Class C	14 years imprisonment, unlimited fine, or both.	2 years imprisonment, unlimited fine, or both.

Exemptions were permitted for doctors, dentists, pharmacists and veterinarians acting in their professional capacity and allowances were also made for scientific research (United Kingdom Government, 1971). An Advisory Council on the Misuse of Drugs (ACMD) was established in order to classify any future drugs (The British Medical Association, 2017). Various modifications have been made to the Misuse of Drugs Act, 1971, with the last one in 2017, which added the synthetic opioid known as U47,700 to the list of Class A drugs; twelve substances (some of which were

previously listed in the ‘temporary’ category) were classified as Class B; and sixteen "‘designer’ benzodiazepines" were added to Class C. These sixteen include: Etizolam (**7**) (4-(2-Chlorophenyl)-2-ethyl-9-methyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a] [1,4]diazepine), Metizolam (4-(2-Chlorophenyl)-2-ethyl-6H-thieno[3,2-f] [1,2,4]triazolo[4,3-a][1,4] diazepine) and Nitrazolam (1-Methyl-8-nitro-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine) (United Kingdom Government, 2017b).

The 1971 Misuse of Drugs Regulations were introduced to clarify the 1971 Act. The regulations helped to outline the circumstances permitting controlled substances to be lawfully supplied, such as through prescription and pharmaceutical dispensation, for use by a named patient only and covered everything from prescription, to storage, disposal and record keeping.

The regulations have been updated over the years with the most recent version being the Misuse of Drugs Regulations of 2001. (United Kingdom Government, 2001). In addition to the classifications outlined in the Misuse of Drugs Act, 1971, the drug regulations group the specified drug substances into schedules which specify how each of the controlled substances must be handled to ensure safe usage. Drug substances listed under Schedule 1 such as cannabis, cathinone, LSD and now etizolam (**7**) are illegal and are prohibited for use. Schedule 2 drugs, which include diamorphine and cocaine, must be stored in a locked safe with a handwritten register of all additions and withdrawals. These drug substances may be prescribed with details of the form and dosage level to be taken. Schedule 3 drugs such as temazepam (**19**), flunitrazepam and phenazepam (**16**) require the same security as Schedule 2 without the handwritten register. The majority of benzodiazepines, including diazepam (**1**) itself are listed as Schedule 4 part 1. These drug substances do not require such a high level of security as those above but are only legal by prescription and are illegal if supplied otherwise, where Schedule 4 part 2 drugs do not require a prescription but must be for personal use. The only requirement for Schedule 5 substances such as is that invoices must be retained for two years. Schedule 5 drugs include preparations of some controlled substances such as codeine and morphine in a prepared and low strength form.

The Psychoactive Substances Act of 2016 was introduced in order to prevent the production, supply, import and export of any psychoactive substances, with the exceptions of caffeine, tobacco and alcohol, not already regulated by the Misuse of Drugs Act (Home Office, 2016).

In the United Kingdom, the manufacturing, distribution, supply and storage of all medicines is monitored by the Medicines and Healthcare products Regulatory Agency (MHRA). In order to manufacture medicinal products, pharmaceutical companies are required to have a Home Office Licence in addition to any MHRA registration documents. Pharmaceutical companies register with the MHRA, stating the product they intend to manufacture along with a list of ingredients, the intended purpose of the medicinal product and potential side-effects. Tablet specifications particular to that company, such as tablet weight and size are also provided. Tablet weight and size may be variable between companies but each company has to register the details of their own product. Each licensed company is then legally obliged to ensure that the tablets meet the stipulated criteria for the entirety of their shelf life. The MHRA ensure that the specifications for the tablets, including dosage level are met, in accordance with the British Pharmacopeia monographs by undertaking regular inspections. (Medicines and Healthcare Products Regulatory Agency, 2012). This means that not only do the MHRA perform inspections and quality checks but pharmaceutical companies also monitor their own products. Therefore, preformulation studies are performed ensuring tablets are stable and have the correct bioavailability; and random samples are regularly analysed to ensure they continue to meet the required standard.

2.5 Illicit Drugs

2.5.1 Substandard and Counterfeit Products

One of the problems within the drugs sector is the rise in the sale of inferior and/or counterfeit medicines. According to the World Health Organisation (WHO) 'Substandard' medicines are authorised products, which do not fulfil the standard of quality expected nationally and/or internationally. Counterfeit or 'falsified' medicine is defined as misrepresenting the true origin or content of the product by deliberate or fraudulent means (World Health Organisation, 2015b). This is a particular problem for people trying to buy medicinal products without a prescription and in countries such as the USA, where medical treatment needs to be paid for. The possibility of buying products over the internet at a cheaper price is very appealing. However, the purchasing of medicinal products is problematic because unless the supplier is known, there is no certainty that the tablets have been pharmaceutically manufactured or are of sufficient quality. This dilemma was discussed by Liang and Mackey (2009), who called for more regulation and accountability for internet sales. They identified the real victims as being the more vulnerable patients including the elderly who may be naïve or less well-off. As a consequence they may receive tablets that are not effective, suitable nor safe and take the tablets with no medical supervision and consequently suffer further financial loss as well as potential harm. It has been suggested that although counterfeit products do exist, the majority of sub-standard drugs on the market are pharmaceutical medications that were not made with adequate quality control (Johnston and Holt, 2014). The problem is exacerbated when the products are bought to treat severe medical conditions or disease. In the UK, the collection of information relating to counterfeit medication, as well as quality control, falls within the remit of the MHRA (Medicines and Healthcare Products Regulatory Agency, 2012).

In 2016, the WHO noted that 920 types of medical product had so far been identified as 'substandard, spurious, falsely labelled, falsified and counterfeit' (SSFFC) (World Health Organisation, 2016). In addition, they warned of the dangers posed by these products, which had been found to contain varying levels of active drug substance, no active ingredient or different drug substances altogether. It was commented that

the falsified products were often unhygienically produced by untrained operators, thus resulting in toxic levels of impurities, or chemicals and possibly having bacteria encapsulated within (World Health Organisation, 2015b).

Although clandestine laboratories are known to produce tablets containing illegal substances such as methylenedioxymethylamphetamine (MDMA), with varying contents (Sherlock *et al.,* 1999), knowledge on which medicinal products have counterfeit counterparts is slower to emerge. In 2014, it was noted that approximately 10% of all drugs on the market were counterfeit (Dégardin, Roggo and Margot, 2014). A great deal of analysis has been performed and recorded about illicitly manufactured anti-malarial tablets (Ricci *et al.,* 2007; Nyadong *et al.,* 2007), as well as the erectile dysfunction drugs Viagra and Cialis (Jung *et al.,* 2012; Bate and Hess, 2010; Kwok and Taylor, 2012; Goldstein *et al.,* 2015). Interest in diazepam (**1**) has grown over recent years, with reports claiming that the majority of the illicit tablets originate in Scotland (Birch, 2012); however, there has been little analytical work performed on these tablets.

2.5.2 The Rise of Illicit Drug Usage in the United Kingdom

During the 1960s, a new generation of heroin addicts emerged. The Brain Committee reported this had resulted from over-prescribing by a small number of doctors. It was believed that the unused prescription drugs had been diverted into the illicit market. The drug problem continued to worsen and from 1975 - 2000, there was an estimated increase in the number of heroin addicts in England, from approximately 5,000 to 281,000. In Scotland there were estimated to be over 50,000 addicts by the year 2000 (Reuter and Stevens, 2007).

Along with the increase in the reported number of heroin addicts came an increase in reported seizures. An interdepartmental government document on tackling drug misuse, along with guidelines for doctors was produced in1998 and updated in 2000 (United Kingdom Cabinet Office, 2000). Although concerns were raised over the social and economic problems associated with drug use, medical issues such as the use of dirty needles leading to an increase in HIV cases, focussed attention on minimising risk.

By the end of the 20th century, a number of new drugs such as amphetamine, LSD, barbiturates and 'party' drugs such as ecstasy (3,4-methylenedioxymethamphetamine) had emerged. These became linked to crime, with a heavy socio-economic impact. Surveys in England and Wales indicated that a third of men in prison and one quarter of those on community service admitted to problematic drug issues in the years 2000 and 2002. It has been estimated that in 2005-6 approximately £508 million was spent on treatment; with an increase in the number of people being treated from approximately 67,000 in 1994 to around 195,400 in 2006-7 (The British Medical Association, 2017).

A government report into the impact of the drug market in Scotland estimated that over £51million was spent on benzodiazepines alone in 2006 (Casey *et al.,* 2009). By 2007, it was estimated that the illicit drug market in the UK was worth over £5 billion but the economic and social costs resulting from drug related crime in England and Wales was estimated to be over £13 billion (Reuter and Stevens, 2007).

An advisory council report in 2016 noted an increase in individuals seeking treatment for addiction to prescription drugs and warned of an increase in diversion of pharmaceutical tablets. However concern and urgent action was requested on the purchase of wholesale powders allowing illicit tablets to be illegally manufactured in the UK (Advisory Council on the Misuse of Drugs, 2016)

2.5.3 Sourcing of Illicit Drugs

A study carried out in the USA in 2014 reported that deceiving doctors was more prevalent than previously realised. Out of 2349 young adults surveyed 4% admitted misleading doctors to obtain prescription drugs, mainly for personal use, though around half also indicated that the ability to sell on the products provided an added incentive (Stogner, Sanders and Miller, 2014). Surveys of patients in America have indicated that the majority of benzodiazepine users purchased their tablets on the street (Jaffe *et al.,* 2004; Stein *et al.,* 2017).

In the United Kingdom, the diversion of pharmaceutical tablets into the illegal supply chain was described by the Advisory Council on the Misuse of Drugs in 2016. The prescription medicines which were most frequently diverted were identified as

benzodiazepines and prescribed opioids. It was also reported that the MHRA had investigated reports on diverted diazepam (**1**) tablets, along with other substances, leading to arrests. (Advisory Council on the Misuse of Drugs, 2016). However, a reduction in benzodiazepine prescriptions may be making the diversion of pharmaceutically manufactured diazepam (**1**) tablets less viable (Johnson, Barnsdale and McAuley, 2016). Data recorded by the Pharmaceutical Security Institute in America indicated that between 2005-10 there was a 66% rise in the number of incidents involving the theft or diversion of pharmaceutical tablets, whereas instances related to counterfeiting increased by 122% (Mackey *et al.,* 2015).

A prevalence of bulk supply purchases over the internet has also been reported (Advisory Council on the Misuse of Drugs, 2016). The accessibility of the internet has provided an easy means for consumers to buy products from their own home without even visiting a doctor. Apart from regular internet sales, the availability of the Darknet allows access to a variety of both illegal and prescription drugs including benzodiazepines. The Global Drug Survey reported that there has been a continuing rise of sales through the Darknet over the last three years (Winstock *et al.,* 2016). It has been noted that many customers believe this to be a safer way of buying illegal drugs with around a quarter of drug sales reported to take place over the internet; a trend that is likely to rise (United Nations Office on Drugs and Crime, 2016; Van Buskirk *et al.,* 2017). Of those surveyed in 2015, 30% claimed to have ingested a wider variety of active substances because of the ability to purchase substances on the Darknet, rather than buying products elsewhere (United Nations Office on Drugs and Crime, 2016).

An investigation into drug trafficking over the Darknet reported that the USA was listed as the country most ‘shipped from’, with 24.9% (11,996) of the 48,026 listings found on the Evolution marketplace. The United Kingdom was listed as the second most ‘shipped from’ country at 12.4% (5972) of all listings. Along with Agora, Evolution traded from January 2014 – March 2015 and was one of the two main Darknet marketplaces at the time the study was being performed. It was also discovered that cannabis accounted for over 25% of the listings, opioids over 15% and benzodiazepines at under 10% of the listings (Rhumorbarbe *et al.,* 2016).

The Darknet investigation was based at the University of Lausanne in Switzerland and part of their research involved making four purchases over Evolution. Prices were believed to be higher than street samples but offered safety and anonymity of online purchases by avoiding physical contact with sellers. The purchases were made from sellers who specified shipment from Switzerland. The results of the investigation reported that the packages arrived by the Swiss postal system in a normal envelope. However, inside the envelope the goods were packaged in heat sealed static shielding bags, to avoid detection (Rhumorbarbe *et al.,* 2016).

Chemical analysis on the samples of cocaine purchased in the Darknet study, showed that one of the samples had the same chemical profile and cutting agents as two cases previously seized by Swiss police. It was therefore implied that the online sample and seized cases originated from the same distribution network (Rhumorbarbe *et al.,* 2016).

2.5.4 Drug Seizures

A study in 2007, commented that law enforcement is focussed on tackling drug supply, by discouraging dealers and making the trade of illicit drugs more difficult. It is hoped that this would lead to rising drug prices and lower illicit drug use. However, it reported that the drug markets in the USA were resilient and despite increasing conviction rates and lengthier jail sentences being put in place, the illicit drug market was thriving. This was partly due to the expendability of those working in the supply chain and the ease of finding replacements, combined with stability in the prices of illicit market drugs (Bouchard, 2007).

The trading of illicit drugs on the street poses problems for vendors. They need to perform their transactions unseen, with customers they can trust. There is also the dilemma of wanting to increase the customer numbers while avoiding becoming recognised as a drug dealer. It was reported that sales would take place in areas with good visibility allowing look-out guards to be posted, or quick street transactions in commercial areas during daylight hours to avoid attracting attention, until mobile phones permitted the arrangement of private meetings. This meant that new

customers were usually only accepted on recommendation (Aldridge and Askew, 2017). However, this remains problematic with police targeting the drug market.

Over the period 2016/17, there were a total of 138,955 drug seizures made in England and Wales by the police force, the transport police and the Border Force. The majority of seizures were Class B drugs, with almost 75% involving cannabis (Broadfield and Marshall, 2017).

Although the total number of seizures was recorded as a 6% decrease on the previous year, it does not account for changes in police activity or records. It also does not reflect the prevalence of the illicit drug trade (Broadfield and Marshall, 2017).

In line with this, there was a 2% decrease in the seizure of Class C drugs, although this was only a fall of 1% in the case of benzodiazepines. The majority of Class C seizures (39%) comprised of benzodiazepines, with a total of 1,945 seizures during the 2016/17 time period. The number of benzodiazepine tablets seized (567,438) was more than double the previous year but was heavily influenced by four different seizures, in South Wales, containing over 30,000 tablets. It was noted however, that seizures made by the Border Force tended to contain far larger quantities than those made by the police. This included 53% of the seizures of anabolic steroids and 47% of seizures containing other Class C drugs, which included the benzodiazepines (Broadfield and Marshall, 2017).

The statistics on seizures in Scotland separate data according to 85-92% of supply cases and estimated figures relating to possession. The data indicates that the majority of seizures in the period 2015/16, involved cannabis. However, the Class C drug seizures were dominated by benzodiazepines, with diazepam (**1**) being reported as the most commonly found. In crimes related to possession, 90% of all cases involved herbal cannabis, cannabis resin, cocaine, heroin or diazepam (**1**). For supply related crimes, 382 out of 393 seizures on Class C drugs, were listed as benzodiazepines and totalled 1,276,100 tablets (Scottish Government, 2017).

Internet sales present different issues. Purchasers and suppliers may be anonymous, making identification of the culprit difficult but they are faced with

problems relating to inconspicuous shipment and delivery. Also there is no protection against misconduct. Trading on the Darknet leaves customers open to exit scams, such as the closure of Evolution in March 2015, when the moderators stole around $12million of buyers money, stored in the system (Van Buskirk *et al.,* 2017).

By using information on forums, the first Silk Road was closed down in 2013. In November 2014, Operation Onymous succeeded in closing down a number of cryptomarkets and several arrests were made (Rhumorbarbe *et al.,* 2016; Aldridge and Askew, 2017). Confronting cryptomarkets has become an essential part of dealing with the illicit drug market (Broséus *et al.,* 2017).

2.6 Analysis of Illicit Diazepam (1) Tablets

According to British law, the possession and use of certain active substances are prohibited and prescription drugs, such as diazepam (**1**) are controlled. The terms of use are detailed under the Misuse of Drugs Act 1971 and the Misuse of Drugs Regulations which are updated as the need arises. Breaking these laws can lead to prosecution, however proof is required to confirm the active drug substance present along with the number of tablets involved in order to substantiate the crime that has been committed. Tablet numbers could help support a supply charge but the circumstances of the case and the fact there is a controlled drug present determine the offence (United Kingdom Government, 1971; M. D. Cole, 2003).

In addition, to providing forensic evidence for prosecutions, analysis of seized drug samples can provide useful intelligence for both police and medical staff. Medically, analysis can help alert doctors to problems that may arise in emergency cases, especially if drugs ingested are not those which the consumer believed them to be. Problem batches of tablets in circulation that have a specific visual appearance and which have been previously analysed, can therefore provide valuable information. Police intelligence can be gained from discovering potential links between cases, which could provide information regarding the origins of illicit substances or help to uncover a drug network (Esseiva *et al.,* 2007).

In the past, it was suggested that if seized tablets appeared to be pharmaceutical products, then literature could be used to support the tablet identity but when the markings were not recognised, full chemical analysis was required. This included presumptive and confirmatory chemical testing (United Nations Office on Drugs and Crime, 2012). However, it is no longer feasible to assume that an official logo is representative of tablets being pharmaceutically manufactured, as many of these markings are now reproduced in clandestine laboratories (Police Scotland, 2016)

When an unknown substance is seized, presumptive tests may be used to give an indication of the active drug substance present and to inform decisions on the type of confirmatory test to be performed (Philp and Fu, 2017).

Although the Zimmerman test has been used as a spot test for benzodiazepines, through the creation of a Meisenheimer complex (El-Hawary, Issa and Talat, 2007; Philp and Fu, 2017), colour tests are not ideal presumptive tests because the results are not specific.

Thin Layer Chromatography (TLC) has also been used as a test for benzodiazepines. Although there is not a development reagent that is specific for benzodiazepines, it can be performed using a sprayed solution of 1M sulphuric acid, which is visualised under UV at 366nm. This is followed by spraying with acidified potassium iodoplatinate, which produces purple spots in the presence of alkaloids by reacting with tertiary and quarternary nitrogen atoms (United Nations Office on Drugs and Crime, 2012; Baerheim Svendsen and Verpoorte, 1983; M. D. Cole, 2003). The method was compared to an Enzyme-Linked Immunosorbent Assay (ELISA) assay for detection of morphine in urine samples of drug abusers. False positives were detected in the assay and it was therefore suggested that the TLC method be used in conjunction with assays as a relatively cheap method of checking for potential errors (Alireza Timcheh-Hariri *et al.,* 2016).

Due to lack of specificity, it is more common to analyse several tablets from one case using GC-MS and comparing the fragmentation pattern and retention time to identify the main drug substance (United Nations Office on Drugs and Crime, 2012).

An investigation into nimetazepam tablets in Malaysia used GC-MS to compare pharmaceutical batches to illicit cases. The results revealed that 11 out of the 64 illicit batches tested (17.2%), were found to contain diazepam (**1**) instead of nimetazepam (Abdullah *et al.,* 2012). Nimetazepam is a benzodiazepine used to treat severe insomnia and was commonly prescribed in Southeast Asia (Manchester *et al.,* 2018). In the UK, nimetazepam is controlled under the Misuse of Drugs Act 1971 as a class C substance under schedule 4 part 1 and was listed as one of the most commonly encountered controlled drugs in 2017 (United Kingdom Government).

A comparison of techniques between a quadrupole mass spectrometer (QMS) and an ion-trap mass spectrometer (ITMS) was performed using diazepam (**1**). This showed that although the ITMS demonstrated greater sensitivity, the ion-mass ratio proved more precise than with the QMS. Full scan analysis was considered more beneficial than examining a smaller range but filtering out the unwanted ions meant that some accuracy was lost (Fitzgerald *et al.,* 1997).

As benzodiazepines are thermally labile, quantification of the active substance is better achieved by High Performance Liquid Chromatography (HPLC) (M. D. Cole, 2003). Until recently, the majority of benzodiazepine tablets in the illicit market were believed to have been pharmaceutically manufactured (United Nations Office on Drugs and Crime, 2012) therefore, much of the literature on HPLC analysis of the tablets has been aimed towards pharmaceutical batch analysis. For example, the HPLC quantification of diazepam (**1**) by Sruthi *et al*. (2013) was performed by crushing twenty tablets together, mixing the powder and then removing 25 mg for analysis. Similarly, an investigation into alprazolam (**2**) tablets, involved crushing ten tablets together for analysis (Pérez-Lozano *et al.,* 2004). This batch analysis is not suitable for illicit tablets as variability between concentrations of the active substance may occur through uneven blending and only an average value for the batch will be achieved.

Further analytical techniques have been recommended by the UNODC, including infrared spectroscopy, although difficulties in extracting the pure drug substance and solubility of derivatives were noted. Although the current UNODC is dated 2012

some of the techniques listed are nonspecific and do not particularly reflect current laboratory best practise.

It is also indicated in the report by UNODC, that any techniques used should always be repeated for comparative purposes, with a known pharmaceutical tablet alongside the unknown sample (United Nations Office on Drugs and Crime, 2012).

The World Health Organisation (WHO) stress the importance of ensuring the quality of pharmaceutical products (Ratanawijitrasin and Wondemagegnehu, 2002), which supports the MHRA in their inspection and regulation of manufacturing processes. Quantification of diazepam (**1**) within tablets is regularly performed for quality control purposes in the pharmaceutical industry, leading to studies which are aimed at enhancing the techniques used (Ferreyra and Ortiz, 2001; Moros, Garrigues and Guardia, 2007). However, even for industrial quantification of active ingredient, tablets tend to be batch tested, with the tablets being ground together. While this technique may be suitable as a quality control check during pharmaceutical manufacturing processes it is not suitable for the analysis of illicit tablets, where each item could be different.

In 2015 the WHO sent out a Medical Product alert relating to falsified diazepam (**1**) tablets which had emerged in Central Africa. The yellow tablets bearing the imprint AGOG were sold in containers labelled as diazepam (**1**) but were found to contain haloperidol (World Health Organisation, 2015b). Instances of fake diazepam (**1**) tablets have also been recorded in Scotland (McGivern, 2016; BBC News, 2017). However, although it has been recorded that ‘diazepam’ (**1**) tablets produced in clandestine laboratories have entered the illegal supply chain little work has been done to identify active ingredients or quantify the level of drug substance present.

2.7 The Use of Statistical Analysis

Physical and chemical analysis of illicit tablets provides a variety of information regarding the content and manufacturing procedure. Due to the different options regarding formulation and tableting methods, there is likely to be significant variation between the physical and chemical characteristics of tablets produced by different illicit manufacturers and more similarity between tablets made in the same

clandestine laboratory. Therefore, knowledge of these differences and similarities can be used to separate illicit cases into different groups. Similarities between chemical profiles can therefore highlight potential links between illicit cases, indicating that they are not isolated incidents (Morelato *et al.,* 2013).

The use of a database allows information to be stored and compared to other cases, maybe from a wider area. This means that whereas a single case of illicit tablets may require investigation for a particular prosecution, similarities to other cases may provide useful intelligence for investigating a bigger problem (Morelato *et al.,* 2013).

Potential links between cases are based on similarities demonstrated by a combination of physical, chemical and statistical characteristics rather than by a single factor (NicDaéid and Waddell, 2005). The characteristics chosen must be clearly defined and be able to distinguish between different batches. Visual analysis of colour is often subjective (Dams *et al.,* 2001) and therefore not ideal for this analysis. Whereas, tablet weight and thickness are definable measurements and provide variable results based on differences in die pressure and the amount of powder added to the die, therefore allowing variation between cases to be observed (Marquis *et al.,* 2008).

Chemical analysis can include GC-MS to explore impurities created during the synthesis of the active drug substance if it makes up a large percentage of the tablet weight (Weyermann *et al.,* 2008; Morelato *et al.,* 2015). A study into processing impurities of diazepam (**1**) led to six impurities being identified and characterised by Kalas *et al.* (2015). Identification of the active drug substances present, given the many types of drug available on the illicit market, along with identification of excipients, can also help to characterise the tablets, particularly if the illicit manufacturer continually uses the same formula (Baer, 2007). Each of these characteristics help to distinguish between different cases of illicit tablets and similarities may therefore be significant.

Comparison and interpretation of results can prove difficult and may be subjective, especially when large quantities of data are generated by a variety of analytical techniques being used to analyse a number of samples. Although statistics may not

provide a definitive solution, it does remove any bias in interpretation of results and is a good way of comparing large datasets.

A variety of statistical methods are available for interpreting the data. In terms of profiling illicit tablets in this study, the aim was to find ways of investigating potential links between cases. Therefore, statistical clustering techniques such as agglomerative hierarchical clustering (AHC) and k-means clustering were an effective method of exploring patterns within the data (NicDaéid and Waddell, 2005). A review by Dams *et al*. (2001) described the benefits of using the chemometric methods of principal component analysis (PCA), AHC and k-means for investigating links between heroin samples. Klemenc (2001) used these techniques and recorded results of between 95-100% accuracy. K-means clustering was also used to characterise two clusters of ecstasy tablets seized in nine different cities in the Sao Paulo district of Brazil, thus identifying links based on the chemical profiles (Maione *et al.,* 2017).

Chemometric techniques can be used to enable prediction of group membership. Once different groupings can be separated, the data can be used to build a training set used to inform on the group membership of future samples. These supervised techniques include linear discriminant analysis, which was used by Johnston and King (1998) to predict the country of origin of seized heroin, based on the concentrations of alkaloids and adulterants within the samples. However, it was recorded that the content of heroin samples changed over time and therefore comparing data from future samples to information stored on a database generated by previous seized samples, may be flawed.

The benefits of creating a database to store information can provide more than local intelligence (NicDaéid and Waddell, 2005). Establishment of a common method of analysis, enabling a means to share intelligence through a common database has been discussed and tried (Weyermann *et al.,* 2008; Lociciro *et al.,* 2008). The illicit drug market has no borders and it has been suggested that cross-border intelligence is valuable in tackling drug crime (Lociciro *et al.,* 2008).

2.8 Assumptions and Strategies used in this Study

Although it has been recognised that counterfeit versions of medicinal products are produced in clandestine laboratories, there appears to be little documentation or research on illicit diazepam (**1**) tablets. A great deal of the work performed on diazepam (**1**) tablets appears to be based on the assumption that they are pharmaceutically manufactured and is concerned about ensuring standards are met or comparing the varying levels of active substance in tablets produced by different companies. Batch testing is often applied to pharmaceutically manufactured tablets however, illicit tablets may show more variability between tablets and therefore need to be analysed individually.

This project focussed on analysing individual tablets taken from 65 different cases of illicit tablets, which were seized in the Tayside area by Police Scotland. These tablets were compared to known pharmaceutical tablets. Data produced from physical and chemical tests was compiled on a database and a variety of statistical tests were performed to create models with the potential to identify similarities and differences which could highlight links between the different cases. In order to carry out the research, certain assumptions and strategies were employed:

1. The Tablets

During this project 1989 tablets, which belonged to 65 different illicit cases and 5 batches of known pharmaceutical tablets were analysed. The illicit and the pharmaceutical tablets were treated in exactly the same way.

2. Assumptions

The assumption was made that the illicit tablets submitted from each case would be similar to each other in terms of physical and chemical properties. This allowed a variety of analytical techniques to be performed using multiple tablets where necessary, instead of trying to perform every test on each individual tablet.

3. Sampling Strategy

On arrival at the University, each batch of illicit tablets were contained in separate plastic bags. Within each bag, all of the tablets from the batch were loose and able to mix. However, to ensure random samples would be selected for analysis, as the tablets were photographed separately, individual bags were labelled, providing a sample number for each tablet. After photographing the tablets, a random number generator was used, in order to determine which number bag each sample would be placed in. This meant that when tablets were taken for analysis, they were already randomly selected. The use of a random number generator was in line with recommendations in the 'Guidelines on Representative Drug Sampling' produced by the United Nations Office on Drugs and Crime (2009).

Chapter 3. Physical Characteristics of the Tablets

3.1 Chapter Summary

The physical weights and measurements of seized illicit tablets and those of pharmaceutical origin were recorded and compared. The aim was to explore differences and similarities, with consideration to acceptable variability as outlined in the British Pharmacopeia. The weight, diameter and depth of all tablets were recorded on a Microsoft access database, enabling the comparisons to be performed and the information used to differentiate between the different tablets.

The colour of the tablets was recorded by photography using a purpose built lightbox.

3.2 Introduction

3.2.1 The Manufacture of Pharmaceutical Tablets

A large variety of prescription tablets are manufactured for the United Kingdom market. New branded products are initially patented, preventing other companies from manufacturing similar formulations until the patent expires. This allows the developing company time to recoup the expense of discovering and introducing the new medication (National Health Service, 2014). Once the patent period is completed, other manufacturers are permitted to produce their own formula for the manufacture of the same generic drug. This may result in variations of colour, weight and dimensions, due to differences in colourants and excipients used. However, each company must license their product, specifying details of tablet content, weight and general appearance (European Medicines Agency, 2000; Medicines and Healthcare Products Regulatory Agency, 2012). At present, twelve marketing authorisation licenses exist for the manufacture and sale of 10 mg diazepam (**1**) tablets in the UK (Goddard, 2018).

Colour and logos on the pharmaceutically made tablets are useful for identifying the manufacturer and drug substance present and are described on accompanying patient information leaflets. However, the type of font and size are not registered characteristics. This means that for 10 mg diazepam (**1**) tablets produced by MA Pharmachem for example, there could legitimately be a variety of fonts and sizes on their tablets (Wesley, 2014). In the United States of America, the Department of Health and Human Services issue guidelines to promote coded imprints on the tablets or capsules themselves, to aid identification (Department of Health and Human Services USA, 2017).

In the United Kingdom, the TICTAC database can be used to identify pharmaceutical tablets and is regularly updated with photographs and information regarding illicit tablets and capsules. The information provided includes the legal standing of the active substance present and details the formula and chemical structure, along with physical characteristics of the product, in order to separate it from other tablets or capsules of a similar appearance. It is estimated that TICTAC is used by over 60,000 professionals from both healthcare and law enforcement sectors (TICTAC Communications Ltd., 2015).

3.2.2 Tablet Quality

Details of drug specifications and regulations determining the quality and consistency of manufactured products are defined in the British and European Pharmacopoeias and are upheld in the United Kingdom, by the Medicines and Healthcare Products Regulatory Agency (MHRA). This ensures the safe manufacture and storage of pharmaceutical drug products.

Consistency in dosage is affected by even distribution of drug substance in the tablet blend and by variation in tablet thickness, which results from over or under-filling dies on the tablet press and the pressure applied to the press. Tablet circumference and diameter are determined by the die measurements and are therefore more consistent. Checks for confirming tablet consistency are described in the pharmacopoeia. The test for uniformity of weight is defined in Appendix XII C of the British Pharmacopoeia. It states that 20 tablets should be randomly analysed and

that from those tablets, no more than two are allowed to deviate from the average weight by more than a set percentage. In addition, none of the tablets are permitted to deviate by more than twice the set percentage. The percentage variation allowed differs according to the average mass of the tablet. The diazepam (**1**) tablets analysed for this project all fell into the category of weight between 80 – 250 mg. Thus allowing a variation percentage of 7.5% (British Pharmacopoeia, 2008; British Pharmacopoeia Commission, 2017b). This means that two of the tablets could go over or under the average weight by 7.5% but not beyond 15%.

An investigation into identification of counterfeit capsules found that the weight of the legitimate pharmaceutical capsules within the study, fell within the range of 315 – 321 mg, with a corresponding relative standard deviation (RSD) of 1%. In contrast, the counterfeit capsules had a much larger weight range of between 224 – 384 mg and an RSD varying from 2 – 40% (Dégardin, Roggo and Margot, 2015).

A study by Zaid, investigating the content of lorazepam (**11**) half-tablets discussed how the correlation between weight and drug uniformity was important because pharmacies are not equipped to check the uniformity of the drug substance. Half tablets are also taken as lower dosage units, with the tablets split on the score line, therefore emphasising the importance of ensuring even distribution of the active ingredient (Zaid *et al.,* 2013).

3.2.3 Physical Appearance of Illicit Tablets

3.2.3.1 Comparison of the Physical Characteristics

Similarities in the physical appearance of tablets can identify potential links between illicit cases. For example, Camargo explored the possibility of using different digital capabilities of photographs to compare colour, shape and texture of tablets. The resulting information was then used to investigate potential links between tablets (Camargo *et al.,* 2012).

3.2.3.2 Manufacturer Logos

Logos could be a useful way to differentiate between batches of tablets. However, it is possible that an illicit manufacturer may have a number of presses or a multi-station press using a variety of dies, which could produce tablets with an appearance similar to one or more legitimate pharmaceutical manufacturer. Potential links between tablets therefore require identification through chemical analysis as well. Alternatively, chemical differences may become apparent between tablets of a similar physical appearance, potentially indicating that different manufacturers are using similar dies.

3.2.3.3 Tool Marks

Another approach to investigating potential links would be to compare tool marks on the tablets. If the same damage mark kept reoccurring within and between cases, it could indicate that the same die had been used. However, it was decided for three reasons that this would not form part of this current research project. The first being the difficulty in recognising a true damage mark from accidental damage and wear that has occurred over the tablet lifespan as well as any differences in the formulation leading to inconsistencies in visual appearance of the tablets. As discussed in Chapter 2 - Introduction (Section 2.2.6.1 Tablet Presses), differences in blend or formula can result in powders sticking to the punch, produce differences in mechanical strength or result in capping or lamination causing a split in the tablet, making consistent damage marks difficult to identify. Thirdly, with increasing complexity of modern illicit tableting operations, it is more likely that a multi-station tablet press could be used.

3.2.3.4 Granularity of Tablets

The granularity of tablets results from formulation, blend and manufacturing procedure employed (Lopatka and Vallat, 2011). Similarities in granularity may therefore help to identify potential links. A study performed in 2011 explored surface granularity though digital photography, which used the grey scale to differentiate shades of colour that represented various granules, facilitating comparison (Lopatka and Vallat, 2011). This technique therefore required consistent digital photographs

of a good quality. Although granularity was considered for this project, it was not pursued because the appearance of granules does not only result from the tablet constituents and manufacturing processes but also from the effects of storage and the consistent blend of the tablet mix, meaning that even tablets from the same batch could appear different.

3.2.3.5 Colour of Tablets

The colour of tablets vary according to the type and amount of colourant used, as well as how well mixed in the colourant is. Consistency of colour gives the appearance of tablets being more evenly blended and therefore, well made. In the United Kingdom, the colourant most often used in pharmaceutical 10 mg diazepam (**1**) tablets is indigo carmine (E132). However, this may be present in powder or lake form, as discussed in Chapter 2 – Introduction (Section 2.2.4.7 Colourants) (Actavis UK Ltd, 2014; Medicines and Healthcare Products Regulatory Agency, 2016b; Medicines and Healthcare Products Regulatory Agency, 2015b; Medicines and Healthcare Products Regulatory Agency, 2014; Medicines and Healthcare Products Regulatory Agency, 2015c). Alternatives, such as the lake dispersed blue 12726 are also used (Medicines and Healthcare Products Regulatory Agency, 2015a).

Colourants may therefore aid in distinguishing between illicit batches of diazepam (**1**) tablets, however this was not pursued for the project as it was believed greater information may be obtained using other analytical techniques, particularly if only a limited number of colourants were used.

3.3 Overview of the Analysis of the Physical Characteristics

The physical characteristics of each tablet were recorded in as much detail as possible and added to a database built with Microsoft Access. The use of a database for storing information related to the analysis of capsules and tablets is becoming more widespread. It provides a structured method of storing detailed information which can be constantly updated, while allowing comparisons of relevant

characteristics to be performed. Databases can even be set up to flag notifications of similarity as new entries are added (Dégardin, Roggo and Margot, 2015).

The intention for creating a database for this project was twofold. Firstly, the information was detailed in order that a selection of characteristics could be extracted from the database, as required. Thus allowing a variety of differentiation criteria to be utilised. Secondly, as many of the tablets were to be destroyed through chemical analysis, it was important to maintain a record containing as much detail as possible for future work.

Many of the physical characteristics, such as colour, font and surface inconsistencies, were best captured with photography. In case of visual comparison of the tablets at a later date, as much consistency as possible was maintained between the photographs (the details of which are described in section 3.3.1.4 Camera Set-Up). Photographs were then linked to the database.

Tablet weights and measurements were recorded and where possible, compared to data supplied by the MA Pharmachem and Wockhardt, in order to differentiate between the pharmaceutical and illicitly manufactured tablets. This was done by adding the recorded information into the database, which allowed a direct comparison of the information relating to tablet markings, weights and measurements.

3.4 Experimental

3.4.1 Photography of Tablets

In order to keep an accurate visual record of each tablet before its destruction, photographs were taken of every tablet received. The diazepam (**1**) tablets were all circular in shape, usually with a flat surface on both sides. However, some of the cases had bevelled edges and a few were convex in shape. In order to record as much variation as possible, photographs were taken of the front, the reverse and from the side.

Initially photographs were taken using an Olympus DSX100 optical microscope, with a 3.6x objective lens and 1.3x zoom, creating a photograph of 1194 x 1194 pixels at 96 dpi. Later intakes of tablets were photographed with a Nikon D5100 camera, using a 36mm extension tube and a 5000K lightbox producing pictures 3696 x 2448 pixels at 300 dpi.

In order to keep the photographs consistent, a 5000K lightbox was constructed. This was then configured on the white balance settings of the camera. The use of a lightbox meant that the light source could be kept even and shadow avoided. It also prevented any influence from external light sources affecting the colour temperature and ensuring consistency over multiple use. The construction was based on the lightbox used in the study on illicit tablet distribution networks by image collection performed by Camargo *et al.* (2012).

3.4.1.1 Light Intensity

The intensity of the light was important for the photographs. As the tablets were photographed alongside a measure, the depth of field needed to keep both in focus. An increase in light helped to keep the image sharp.

While Digital Single Lens Reflex (DSLR) cameras can boost the brightness of a picture this is usually done at the expense of the quality. In order to reduce any grain and to retain the detail of the surface photographed, the lowest International Standards Organisation (ISO) setting of the camera was used (Brady, 2017).

The other benefit with using greater light was that a higher shutter speed could be used. This helped to reduce the impact of external vibration or “camera shake”, which is more noticeable in highly magnified subjects.

3.4.1.2 Materials for the Light Box

Black Plastic Box (MB7 177 x 120 x 83 mm), 2 x PCB Mounting 4 AA Battery Boxes with mounting screws, 16/0.2 mm Stranded Copper AWG 16/32 3 Amp Equipment Wire 10 mm (black and red) and a Snap-in Miniature Round Rocker Switch (Black) were obtained from Maplin, Edinburgh. 8 x 48 LED 5000K Panels, Size 39 x 48 mm (Part No. SKU014825 – 25210 EVU) and a dual flash bracket holder for studio light

stand tripod camera DSLR 1/4" Screw were purchased from ebay. A plastic cat litter tray (1 mm thick black) was bought from Asda, Dundee and cut to size to mount the LED panels on. Roof bolts (2 x M6 25 mm), dome nuts (2 x M6), spring and flat washers (2 x M6) and 8 x AA Alkaline Batteries were purchased from Homebase, Dundee.

3.4.1.3 Construction of the Light Box

A circle was drawn around the camera lens, in the centre of the top of the black box. A hole was cut which was approximately ¾ inches larger diameter than the drawn circle maintaining a 5 mm distance from the edge of where the camera bracket was to be mounted. The 5 mm clearance allowed for the box and LED panel thickness.

The camera bracket was fastened onto the long face of the box with the M6 roof bolts. The 1mm black tray was cut to size and the LED panels were mounted with the electrical connections towards the lens aperture to allow the assembly to be withdrawn for periodic battery changes.

The four AA battery boxes were screwed to each short end on the inside of the box, allowing a 3 mm clearance from the base lid to allow the box to fully close. Then a 20 mm hole was drilled to position the rocker switch away the components. The two AA battery boxes were soldered in series to create a 12V source and the LED panels in parallel via the rocker switch to the battery boxes (i.e. all positive terminals combined and similarly the negative terminals). Finally, the eight AA batteries were fitted and the panels were replaced ensuring the wires remained away from the lens aperture.

A sheet of photographic paper was printed black, with a 2.5 mm square left white. The sheet was cut to size, locating the white square towards one corner. This was fixed onto the inside of the lid of the black box, which was used as the base. Alongside this, a corner scale was fixed alongside it, to allow measurements to be taken. Images of the construction and set-up of the light box can be seen in Figure 3.1.

Figure 3.1. The purpose built light-box. Showing the light box turned off, allowing the LED lights inside to be seen; switched on, to show brightness; from underneath to show construction; showing the scale and background mounted on the lid; and as seen by the lens; how the camera is fixed to the lightbox and the lens from underneath.

3.4.1.4 Camera set-up

A Nikon D5100 camera with a standard lens was set to 55mm and the filter was removed to prevent reflection. A 36mm extension tube was attached.

The camera was set to manual, with an aperture of F36 and a shutter speed of 0.5 seconds. The ISO was 100 and whitebalance of 5, for the 5000K colour temperature. The quality used was Raw-F (large) for high resolution and spot metering was chosen to focus on a mark or edge of the tablet.

Once the settings had been adjusted, the camera was mounted on the bracket.

3.4.2 Tablet Markings

Before analysis each tablet was physically examined and a brief description of the colour was recorded, along with the tablet markings. Although a note was made of the colour of the tablets, it was acknowledged that this was subjective. However, descriptions such as mid blue or bright blue could be used to give an indication of colour. Differences in colour were best recorded through keeping methods of photography as consistent as possible. The photographs taken in the light box were taken against a black background with a small white box measuring 2.5 mm in one corner. This was to ensure that the white balance was kept constant. This methodology was based on a study by Camargo et al. (2012).

Each tablet was checked for surface irregularities, such as chipping or double strikes and any noticeable detail was recorded. Noticeable differences in font size and style between some cases bearing the same marking, were apparent. Initially, it was believed this may be an area worth investigating. However, after discussions with MA Pharmachem, it was discovered that no specification was listed on the manufacturing licence from the Medicines and Healthcare products Regulatory Agency (MHRA) (as described in section 3.1.1 The Manufacture of Pharmaceutical Tablets).

3.4.3 Tablet Measurements

3.4.3.1 Weight of the Tablets

Due to the stringent rules regarding tablet weight that are laid down in the British and European Pharmacopoeias, weights were recorded and assessed in comparison to the prescribed criteria (British Pharmacopoeia, 2008).

The tablets were each weighed three times on a calibrated Mettler Toledo analytical balance. The mean weight of each tablet was recorded, in order to reduce variation. The weights were noted and the mean, standard deviation (SD) and relative standard deviation (RSD) were calculated for each case. This was compared to the ±7.5% difference permitted and an allowance for any errors of measurement. The error of measurement of the independently calibrated Mettler Toledo was estimated

at ±0.1 mg. Ten weights were recorded of the same tablet, giving a standard deviation of 9.48×10^{-4} mg.

As tablet weight was originally determined by each pharmaceutical company and registered for their own product, it varied between manufacturers. For example, MA Pharmachem manufacture tablets within the weight range 165-175 mg (Wesley, 2014), whereas tablets produced by Wockhardt are noticeably lighter, with the average weight of Wockhardt pharmaceutical tablets analysed for this project being around 153 mg. The variation in weight results from differences in excipient content because although the amount of active drug substance would have been the same, the exact formulations could vary. Therefore, the allowances in weight described for this project were linked to the measured tablet weight and compared to the pharmaceutical criteria.

3.4.3.2 Dimensions of the Tablets

The diameter and the depth of each individual tablet were measured using digital callipers. The callipers with an estimated error of measurement of ±0.02 mm, were calibrated against a series of metric 'Matrix' steel slip gauges by the Coventry Gauge & Tool Company Ltd. The diameter and depth of the tablets were measured across the centre, in line with any markings. Each measurement was taken three times and the mean was calculated, to minimise any variance. The mean, standard deviation and relative standard deviation were then calculated for each case.

The tablet diameter was taken across the centre of the tablets, in line with any markings, where appropriate. However, although lifted from a flat surface, the callipers did not always touch the tablets in the same place on both sides, meaning that instead of being taken directly across the tablet, measurements may have been taken at a slight angle, as demonstrated in Figure 3.2. Any inaccurate measurements were avoided as much as possible and all measurements were taken three times, with the mean being the recorded result to minimise variation.

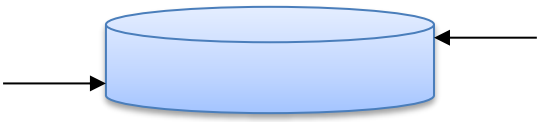


Figure 3.2. Diagram showing position that the callipers could have measured the diameter of tablets.

Despite the potential inconsistency in measurement, due to human errors of measurement, little difference was demonstrated between the diameters within each case, with a relative standard deviation not exceeding 0.56%. Combined with the error of measurement allowance of 0.02 mm, variations in diameter within each case were of little significance. However, because manufacturers re-use their dyes for producing multiple tablets, this was not unexpected. Therefore, the analysis of measurement focuses more on the differences in the depth of the tablets.

Tablet depth was measured around the centre of the tablet and may have suffered from the same problems of measurement as the diameter but this was less of an issue because it was easier to ensure the tablet was held evenly in the callipers.

There was more variation in the depth of the tablets because this was affected by the flow rate and consistency of the tablet blend and the pressure of the tablet press. As there is little deviation in the diameter, it is predominantly differences in formula, thickness and blend that effect consistency in dosage level and weight of the tablets.

3.5 Results and Discussion

3.5.1 Photography of the Tablets

The photographs taken managed to capture much of the detail on the tablets, as shown in Figure 3.3.

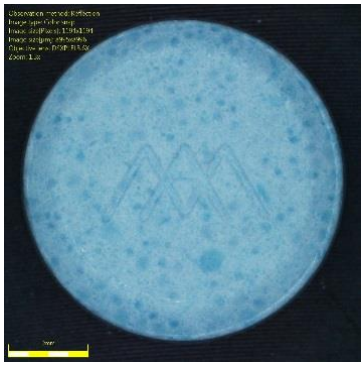
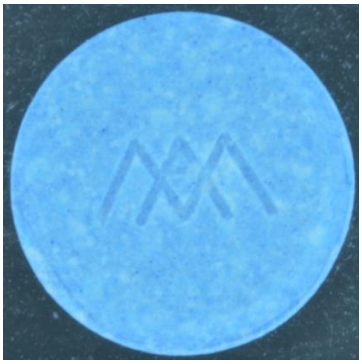
Case 2	Case 76
	

Figure 3.3. Photographs taken by different cameras. Case 2 was taken on the Olympus DSX100 and Case 76 was taken using the Nikon D5100. Difference in colour is genuine.

The main difficulty with the photographs was related to inconsistency with the lightbox. As the lightbox had been designed to be portable and easy to use anywhere, batteries were fitted. However, it soon became apparent that consistency of colour was dependent on the battery life and the tablets became more blue in colour as the battery life reduced. Therefore the photography had to be closely monitored. In hindsight, to avoid battery degradation, an external regulated 12v mains power supply should have been fitted to maintain the power level.

3.5.2 Tablet Markings

3.5.2.1 Imprint Details on the Tablets

The illicit tablets all bore the same logo within each case. Altogether, 15 types of logo were represented by the different cases. The markings related each case are shown in Table 3.1. Colour was also found to be consistent within each case.

Table 3.1. Table identifying the number of cases and the case numbers which bear each logo. The markings on the front and reverse of each tablet are separated by the | symbol.

Logo	Number of Cases with the Logo	Case Numbers
MA | D/10	24	2, 4, 5, 13, 14, 16, 24, 25, 26, 29, 30, 31, 73, 74, 75, 76, 79, 82, 83, 86, 130, 132, 135, 159
Half Score	1	3
Cox | DC	7	6, 7, 8, 27, 71, 72, 150
STADA | D/10	4	9, 12, 28, 129
CP | D/10	2	10, 81
ROCHE 10 | half score	1	11
MSJ | half score	3	23, 78, 90
1|0 | plain reverse	10	77, 84, 85, 87, 88, 89, 131,134, 137, 152
TENSIUM | half score	1	80
MSJ | plain reverse	4	127, 128, 153, 154
C/DC | plain reverse	1	133
Half score | plain reverse	1	136
EZ/1.0 | plain reverse	4	151, 155, 157, 160
10 | half score	1	156
NTZ/1.0 | plain reverse	1	158

The tablet markings MA | D/10 and Tensium are known to relate to pharmaceutical companies who produced tablets for the UK market. Although MA 10 mg diazepam (**1**) tablets have not been in production since November 2012 (Wesley, 2014), they may have still been legitimately available at the time of seizure due to the length of shelf life. However, the imprint CP | D10 relates to products manufactured by Wockhardt UK, who are still licensed to manufacture diazepam (**1**) products.

Cox | DC is not a recognised imprint for diazepam (**1**) tablets licensed for the UK market, nor are those marked with EZ or NTZ. STADA is a German pharmaceutical company and MSJ are based in Sri Lanka, neither of these companies manufacture tablets for the UK and neither do the Swiss company Roche. Therefore tablets bearing logos from these companies are not licensed for the UK market. Similarly, Case 3, which bears a half score and is convex in shape was identified as containing 10 mg of diazepam (**1**) by HPLC analysis (Chapter 5) but does not correspond to any products licensed for the UK. It may be a pharmaceutical product licensed in another country but the lack of detail imprinted on the tablets makes them difficult to identify.

Tablets marked 1/0 were found to contain phenazepam (**16**) by GC-MS analysis (Chapter 4) which is not a licensed product in the UK and therefore these tablets are clearly illicit.

3.5.2.2 Damage Marks on the Tablets

Damage marks including small areas of damage to the circumference on each of the tablets were noted when appropriate. Chips were recorded in conjunction with tablet weight to ensure that 'partial tablets' were not included in the analysis of measurements. In addition, possible damage marks and double strikes were noted, as can been seen in Figure 3.4. Multiple tablets were found to have double strikes in cases 13, 25, 26, 31 and 159. However, further investigation as to the significance of these physical marks were not carried out.

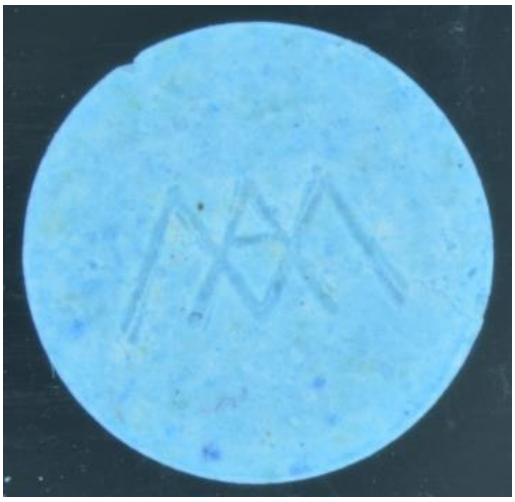


Figure 3.4. Tablet from Case 159, showing a double strike.

3.5.3 Measurements of the Tablets

The results of the measurements are shown in Table 3.2 and are summarised as a bar chart in Figure 3.5 and as scatter graphs and box plots in Figures 3.6 and 3.7. Table 3.2 lists the mean diameter, depth and weight of every illicit case and batch of pharmaceutical tablets, although allowance needs to be made for the ±0.02mm error of measurement for diameter and depth and the ±0.1mg error of measurement for weight. In addition, the relative standard deviations (RSD) are listed for the depth and weight of each case.

Table 3.2 also lists Roche next to the Pharmaceutical tablets. Although the Roche tablets were pharmaceutically manufactured, they were not produced for the UK market. However, with similar work on pharmaceutical intelligence being performed in other countries, Roche tablets may be used for comparative work elsewhere and it has been noted that there is little variability between Roche products manufactured either on one site or in multiple locations and they remain consistently within pharmaceutical guidelines (Dégardin, Roggo and Margot, 2015).

Table 3.2. The mean measurements and weights of each case of tablets. Relative Standard Deviation (RSD) is also given for the Depth and the weights of each case. However, there was a large variation in case size. Where there is a blank, not enough tablets were available in the case to calculate the data. An RSD of zero indicates there were multiple tablets but no deviation.

Case No	Diameter (mm)	Depth (mm)	Depth range (mm)	RSD of depth %	Weight (mg)	Weight range (mg)	RSD of weight %
2	8.08	2.64	2.64	0	170.2	167.6 – 172.6	1.21
3	7.99	3.55	3.51 – 3.57	0.91	206.6	198.1 – 212.1	3.00
4	8.07	2.65	2.63 – 2.66	0.80	220.5	215.0 – 225.9	3.50
5	8.13	3.11	3.07 – 3.15	1.82	216.3	213.6 – 218.8	1.20
6	8.08	2.90	2.83 – 2.97	3.41	189.5	187.6 – 193.2	1.70
7	8.15	3.10	3.10 – 3.47	6.23	181.4	181.4	
8	8.09	3.13	2.79 – 3.46	15.16	181.7	171.5 – 188.5	4.95
9	8.13	3.14	2.82 – 3.45	14.21	195.7	179.1 – 226.0	13.50
10	8.06	2.88	2.88		160.2	158.5 – 161.9	1.50
11	8.03	2.65	2.64 – 2.66	0.53	172.2	172.2	
12	8.08	2.85	2.82 – 2.88	1.49	177.6	175.3 – 179.4	1.20
13	8.08	2.78	2.74 – 2.83	1.65	173.6	171.5 – 176.6	1.30
14	8.16	2.64	2.61 – 2.66	0.96	213.3	185.6 – 228.9	11.3
16	8.07	2.58	2.57 – 2.60	0.59	168.9	168.3 – 171.6	0.46
23	5.53	2.80	2.52 – 2.96	8.61	74.6	71.7 – 77.6	0.1
24	8.09	2.59	2.58 – 2.61	0.67	169.7	169.7 – 170.3	0
25	8.13	3.08	3.07 – 3.09	0.46	214.2	214.2	
26	8.07	3.45	3.44 – 3.45	0.17	181.5	174.1 – 186.9	1.64
27	8.06	3.23	3.12 – 3.45	5.90	194.2	192.6 – 197.4	1.39
28	8.05	2.79	2.74 – 2.84	2.53	178.6	173.8 – 181.7	1.90
29	8.09	2.58	2.57 – 2.59	0.39	174.3	169.3 – 178.4	3.29
30	8.13	2.61	2.60 – 2.62	0.44	170.4	169.9 – 170.8	0.17
31	8.14	3.17	3.03 – 3.46	7.82	181.0	176.7 – 185.4	1.90
71	8.12	2.88	2.77 – 3.03	3.34	171.6	153.7 – 182.1	4.29
72	8.12	3.04	2.96 – 3.18	1.91	185.6	159.7 – 197.7	2.89
73	8.11	2.85	2.81 – 3.00	1.90	181.1	172.9 – 194.2	1.78
74	8.10	2.64	2.59 – 2.72	1.28	169.6	149.7 – 173.3	2.85
75	8.13	2.90	2.76 – 2.97	2.13	181.8	169.0 – 223.5	7.89
76	8.13	2.89	2.82 – 2.99	1.92	180.7	173.5 – 186.5	1.80
77	8.08	2.68	2.64 – 2.75	1.12	193.9	185.4 – 197.5	1.87
78	5.59	2.42	2.37 – 2.54	1.59	73.8	70.2 – 76.3	2.13
79	8.09	2.68	2.63 – 2.78	1.87	170.1	167.3 – 172.6	0.90
80	8.04	2.67	2.59 – 2.77	1.95	170.1	162.6 – 180.1	2.32
81	8.06	2.29	2.23 – 2.71	2.02	153.7	150.5 – 158.9	2.50
82	8.11	2.61	2.58 – 2.65	0.73	170.1	168.4 – 172.1	0.76
83	8.08	2.67	2.61 – 2.71	1.04	169.4	166.1 – 173.4	1.10
84	8.08	2.81	2.69 – 3.01	2.83	244.2	222.6 – 268.1	4.41
85	8.09	2.80	2.74 – 2.89	2.44	242.5	234.7 – 255.3	3.21
86	8.09	2.66	2.60 – 2.73	1.45	169.2	165.3 – 172.7	1.23
87	8.06	2.66	2.64 – 2.68	1.55	194.0	185.0 – 204.3	3.34
88	8.10	2.66	2.65 – 2.69	0.75	198.4	293.6 – 203.5	2.50
89	8.07	2.65	2.63 – 2.66	0.42	197.0	188.8 – 200.1	1.70
90	5.56	2.38	2.32 – 2.43	1.50	73.4	70.3-75.5	2.50
127	7.12	3.33	3.30 – 3.37	0.81	141.7	139.8 – 142.9	0.64
128	7.12	3.31	3.07 – 3.36	2.07	140.6	138.2 – 142.1	0.75
129	8.11	2.89	2.76 – 3.01	2.97	168.9	157.4 – 173.6	2.56

Case No	Diameter (mm)	Depth (mm)	Depth range (mm)	RSD of depth %	Weight (mg)	Weight range (mg)	RSD of weight %
130	8.10	2.92	2.86 – 3.06	1.89	179.8	172.9 - 196.0	3.32
131	8.08	2.78	2.66 – 2.89	2.42	240.4	231.0 – 259.0	3.21
132	8.12	3.07	2.98 – 3.17	1.80	195.0	178.7 – 208.5	3.46
133	8.22	2.85	2.74 – 3.24	4.98	170.5	159.1 – 175.8	2.94
134	8.08	2.61	2.41 – 2.82	4.05	223.3	201.5 – 247.2	5.74
135	8.15	2.89	2.81 – 2.98	1.61	179.1	173.5 – 18.36	1.65
136	8.12	2.94	2.82 – 3.06	1.97	197.0	186.1 – 212.9	3.39
137	8.06	2.81	2.69 – 3.06	4.09	234.8	227.8 – 253.4	3.85
150	8.10	2.87	2.74 – 3.06	2.90	174.1	160.6 – 191.5	4.99
151	8.09	2.83	2.78 – 2.87	0.95	178.7	165.1 – 186.0	4.57
152	8.15	2.70	2.60 – 3.03	3.23	234.7	225.1 – 262.1	3.40
153	7.11	3.31	3.17 – 3.38	1.65	141.5	138.6 – 143.8	0.88
154	7.11	3.32	3.26 – 3.37	0.94	141.2	139.0 – 143.7	1.27
155	8.11	2.88	2.78 – 3.09	2.80	185.3	174.0 – 191.1	2.25
156	8.07	2.52	2.48 – 2.54	0.83	174.2	171.2 – 176.1	0.88
157	8.10	2.89	2.76 – 2.96	2.30	181.9	177.5 – 186.0	1.84
158	8.10	2.81	2.59 – 3.00	2.87	176.5	152.9 – 187.6	5.66
159	8.16	2.90	2.78 – 3.07	2.50	174.4	162.8 – 185.5	3.65
160	8.11	2.83	2.73 – 2.88	1.47	181.6	177.1 – 185.8	1.52
Roche	8.11	2.58	2.57 – 2.60	0.51	169.7	168.3 – 171.2	0.62
MA 061	8.08	2.59	2.55 – 2.63	0.62	170.7	168.1 – 173.0	0.66
MA 064	8.07	2.65	2.61 – 2.70	0.78	170.3	166.9 – 173.6	0.93
Actavis	8.10	2.71	2.66 – 2.78	0.92	173.6	168.6 – 178.6	1.66
Teva	8.10	2.59	2.52 – 2.67	1.17	175.7	167.1 – 179.0	1.14
Wockhardt	8.07	2.28	2.21 – 2.41	1.90	153.6	148.3 – 163.3	1.90

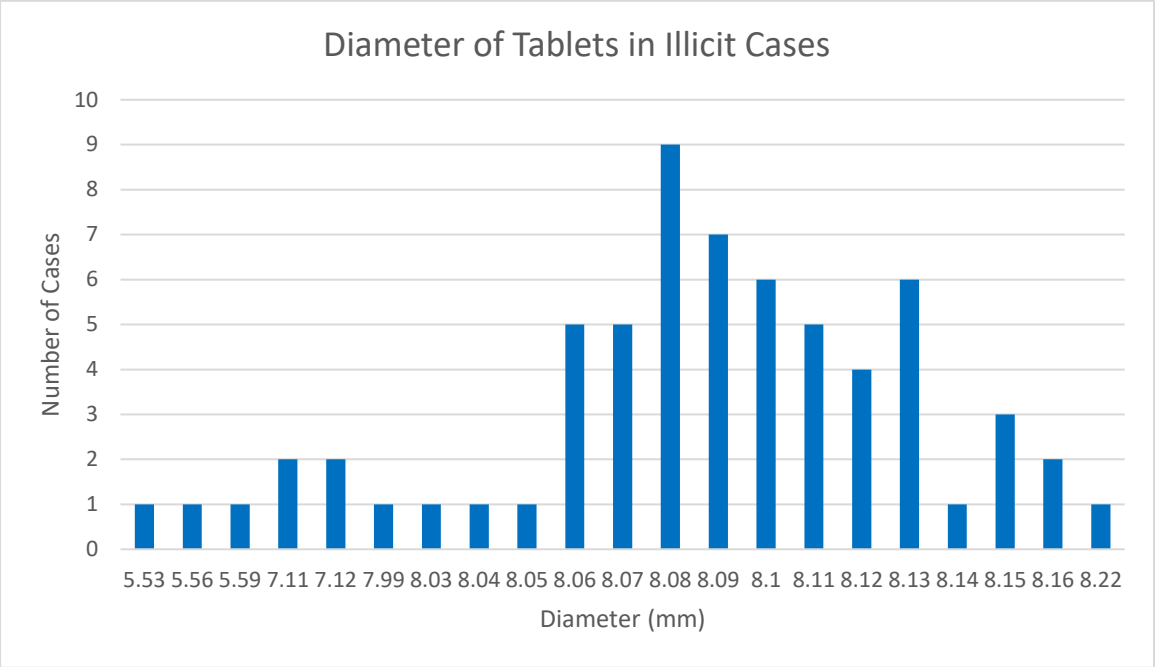


Figure 3.5a Bar chart showing the range of diameters measured from illicit cases of 'diazepam' (1) tablets.

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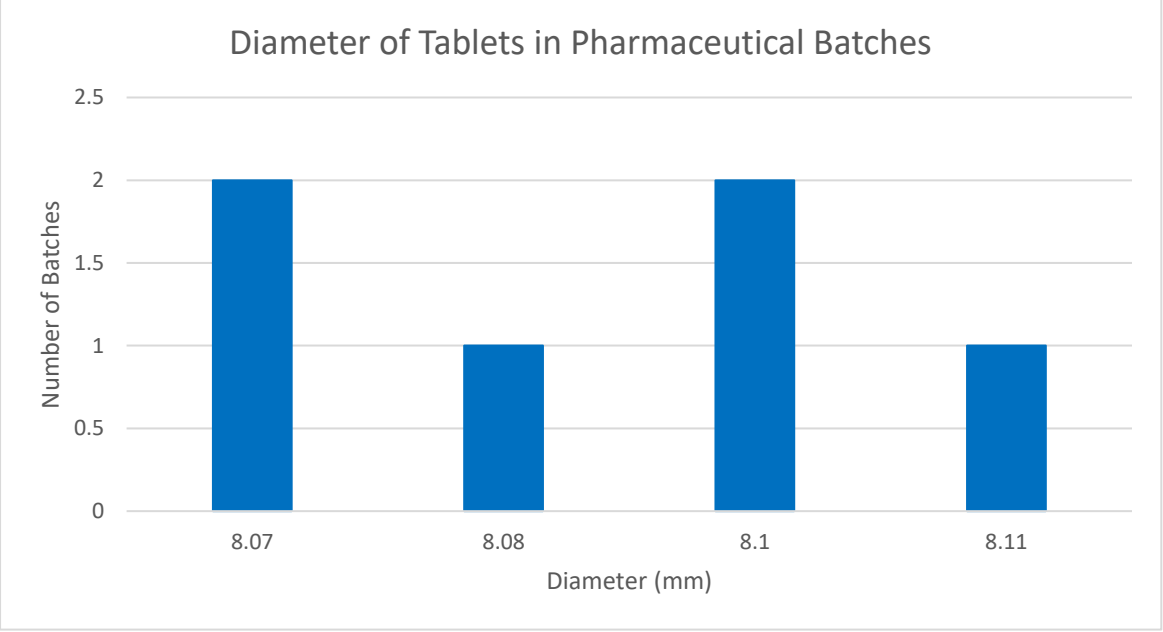


Figure 3.5b Bar chart showing the range of diameters measured from pharmaceutical batches of diazepam (1) tablets.

Figure 3.5 Bar chart showing the diameters of illicit and pharmaceutical batches of tablets.

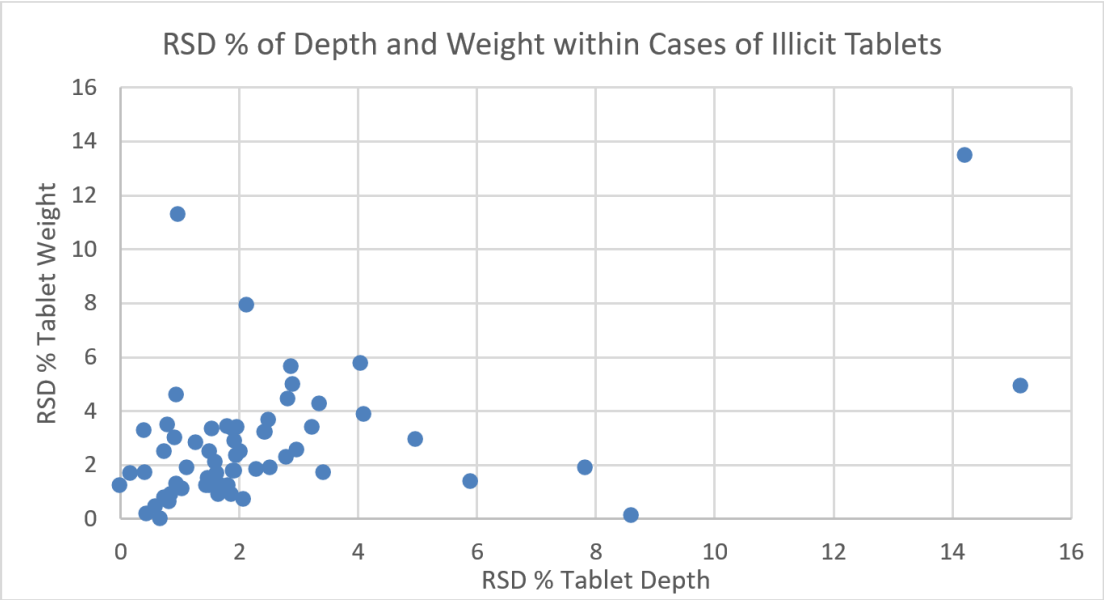


Figure 3.6a The RSD of depth and weight within cases of illicit tablets.

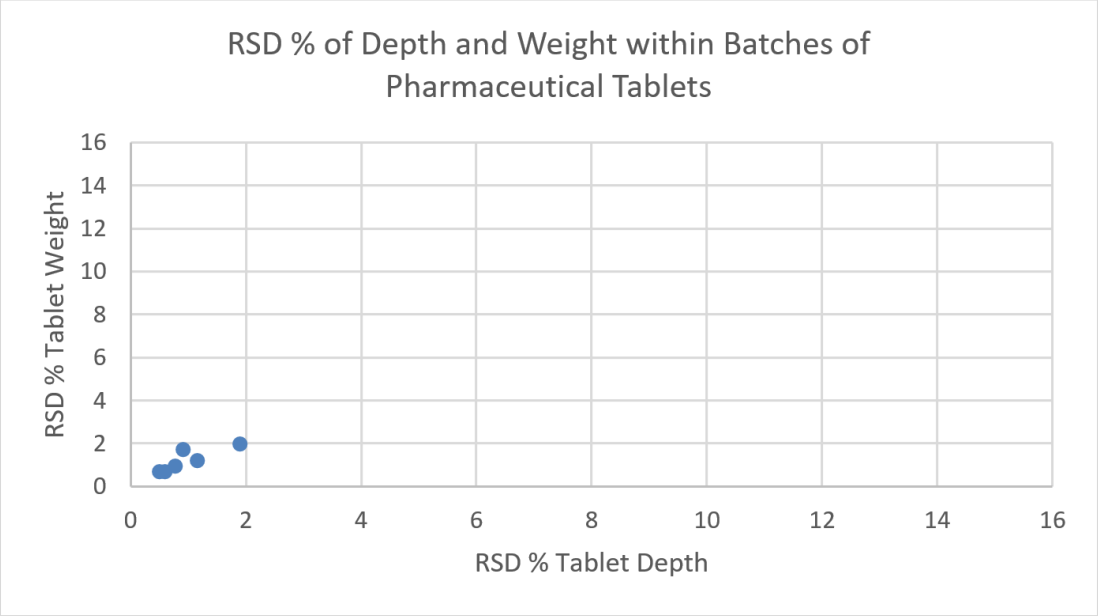


Figure 3.6b The RSD of depth and weight within cases of illicit tablets.

Figure 3.6 Scatter graphs comparing the Relative Standard Deviation (%) in depth and weight within batches of illicit and pharmaceutical tablets.

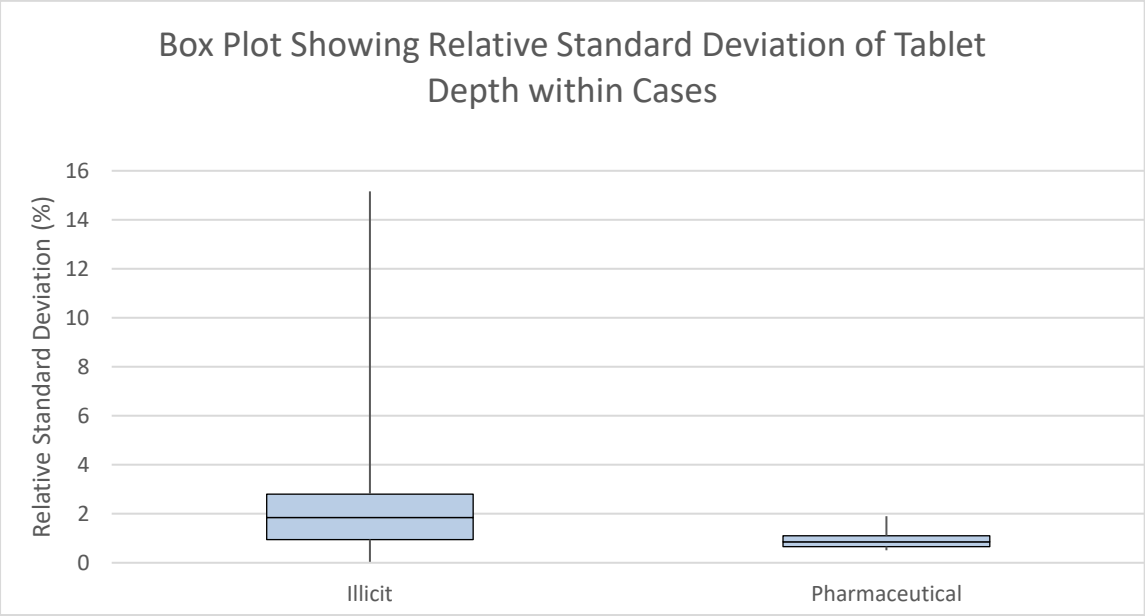


Figure 3.7a Box plot comparing RSD of tablet depth between illicit and pharmaceutical batches of diazepam (1) tablets.

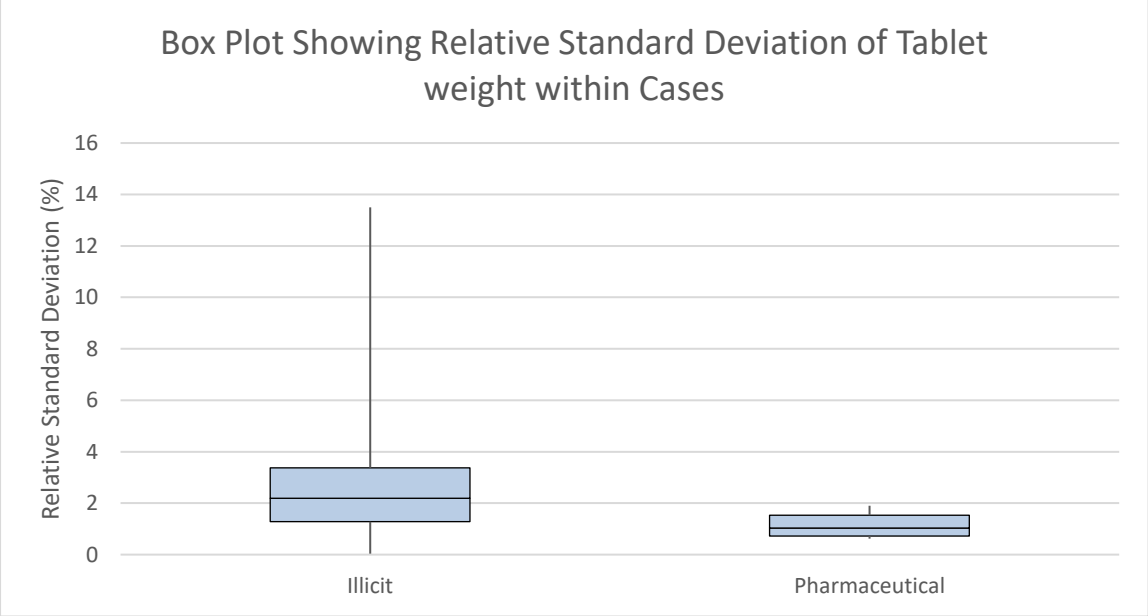


Figure 3.7b Box plot comparing RSD of tablet weight between illicit and pharmaceutical batches of diazepam (1) tablets.

Figure 3.7 Box Plots showing the range of Relative Standard Deviation (%) in depth and weight within batches of illicit and pharmaceutical tablets.

3.5.3.1 Tablet Diameter

As shown in Table 3.2 and Figure 3.5 all of the pharmaceutical tablets had a diameter measuring between approximately 8.05 - 8.12 mm, with little variation due to the fixed size of the dyes used and allowing for errors of measurement.

In comparison, there was much more variation between the measurements generated by the illicit tablets. Cases 23, 78, 90 had a much smaller diameter at around 5.5 mm. These tablets were all marked MSJ, with a half score on the reverse and were found to contain approximately 9 – 10 mg diazepam (**1**).

Cases 127, 128, 153 and 154, also bore the MSJ marking but with a plain reverse, all had a diameter measuring around 7.11 – 7.12 mm, a depth of between 3.31 - 3.33 mm and mean weight of 141 mg. The highest RSD for these four cases was 1.74% for the weight of Case 128 and 2.07% for the depth of the same case however, it is worth noting that this is still within the ±7.5% range permitted within the pharmaceutical industry. Notably, these tablets were also marked MSJ but were coated, convex shaped tablets that were very different in appearance to Cases 23, 78 and 90. However, analysis by gas chromatography – mass spectrometry (GC-MS) later revealed that these tablets contained promethazine as the active drug substance.

Visually the two groups of tablets with the MSJ marking were well made. It was the markings on the tablets and difference in shape and size that identified these tablets as being illicit for the UK market. However, they do have the potential of being pharmaceutically manufactured for use in other countries, since it is not possible to check the markings of all legitimate tablets produced for the rest of the world markets. However, research has shown that MSJ Industries is a subsidiary company of J.L. Morison Son & Jones (Ceylon) plc., which is a legitimate pharmaceutical company producing tablets in Asia and Scandinavia (Substance.org.uk, 2017). However, information regarding the types of tablets manufactured is difficult to obtain.

All of the remaining illicit cases appear to be much closer in diameter to the known pharmaceutical tablets. Case 3 was the smallest, with a diameter of approximately 7.99 mm and Case 133 was the largest with a diameter of about 8.22 mm (Figure 3.8). In comparison, the pharmaceutical tablets measured in the range of around 8.07 - 8.10 mm. In total 76.9% (50 cases) of all seized cases (including those marked MSJ), fell within the range of the measured pharmaceutical tablets. This number includes all cases within ±0.04 mm of the recorded diameter to allow for errors in measurement of both the pharmaceutical and illicit tablets (therefore in the range 8.03 – 8.13 mm).

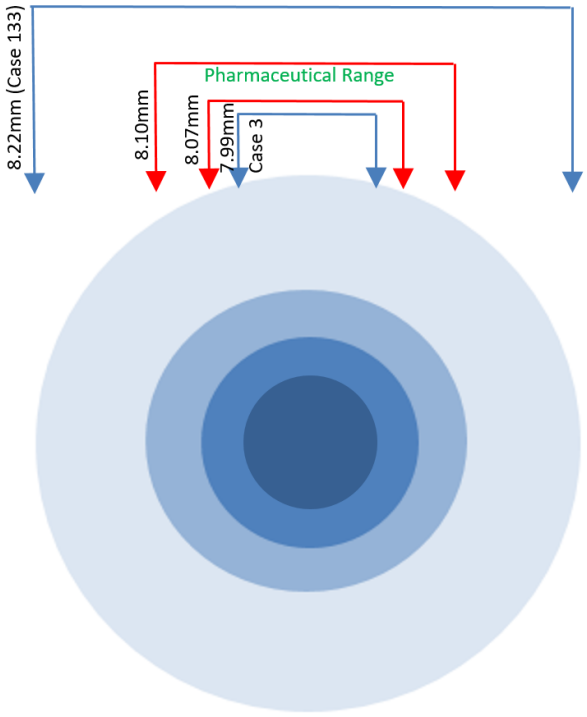


Figure 3.8. Diagram illustrating variation in mean diameter of the cases analysed (excluding the cases marked MSJ which had a mean diameter of around 5.5 mm). All of the pharmaceutical batches were recorded between 8.07 - 8.10 mm which is shown in red. The outer and inner measurements of 8.22 mm and 7.99 mm represent the cases with largest and smallest diameters measured. The diagram is not to scale.

3.5.3.2 Tablet Depth

The difference in range of tablet depth is visualised in Figures 3.6 and 3.7a. The whiskers of the box plot (Figure 3.7a) highlight the large difference in RSD range, while the lower median and smaller variability recorded from the pharmaceutical tablets is also demonstrated.

The depth of the pharmaceutical tablets varied in range from approximately 2.59 - 2.71 mm, with the exception of the tablets manufactured by Wockhardt which had a mean depth of about 2.28 mm. The Wockhardt tablets were the thinnest of all cases, with Case 81 measuring a similar size given the potential error of measurement of ±0.04 mm and showing a recorded mean thickness of approximately 2.29 mm. Interestingly the marking of CP | D10 present on the tablets in Case 81 is consistent with the logo used by the pharmaceutical company Wockhardt UK.

The MSJ marked cases 90 and 78 were the next smallest tablets measuring approximately 2.38 mm and 2.42 mm respectively. These cases were discussed above in section 3.4.3.1 (tablet diameter) regarding their potential to be pharmaceutically manufactured outside the UK for a foreign market. However, in this instance, Case 23, which had a comparable diameter to cases 90 and 78, was recorded as having a greater mean depth of around 2.8 mm, which presents a potentially significant variation.

The pharmaceutical batch with the largest mean depth were manufactured by Actavis and had a mean thickness of approximately 2.71 mm. 41 illicit cases had a recorded mean depth above this, with only 36.9% (24 cases) falling within the range 2.24 – 2.75 mm and allowing for the ±0.04mm error of measurement (Figure 3.9). The case with the largest mean depth was Case 3, measuring approximately 3.55 mm.

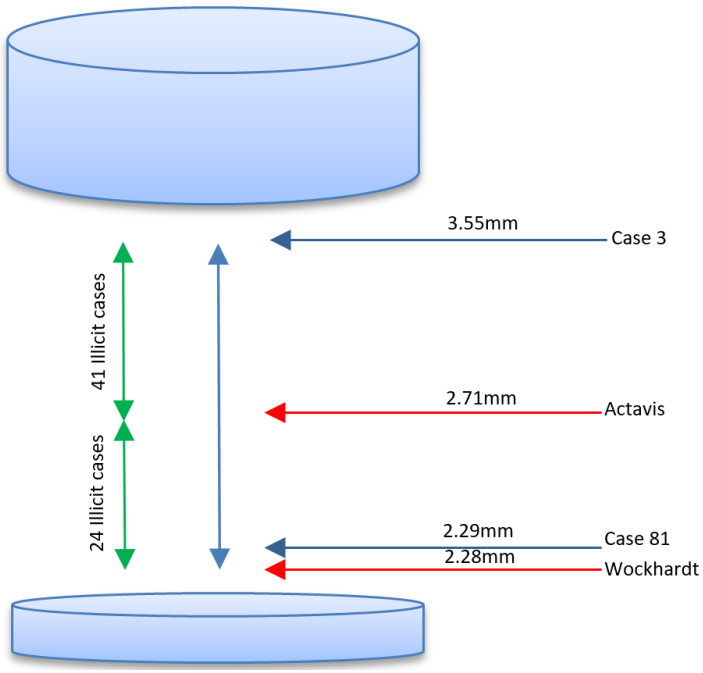


Figure 3.9. Diagram illustrating variation in mean depth of the cases analysed. All pharmaceutical tablets fell between the two red lines marked by the Actavis and Wockhardt batches. Twenty-four of the illicit cases also fell within this range but the majority (41 cases) appeared to be deeper tablets than the pharmaceutical products made for the UK market, that were measured within this project. The diagram is not to scale.

3.5.3.3 Tablet Weight

The difference in range of tablet weight is visualised in Figures 3.6 and 3.7b. The whiskers of the box plot (Figure 3.7b) demonstrate that greater variability was measured between the illicit tablets, while the lower median of the pharmaceutical tablets is also shown.

The known pharmaceutical tablets all weighed between approximately 153 – 176 mg. The heaviest were the tablets produced by Teva with a mean weight of approximately 175.7 mg and the lightest were the tablets manufactured by Wockhardt, which were considerably thinner and had a mean weight of about 153.6 mg. Based on the mean weight of pharmaceutical tablets manufactured by Wockhardt and Teva to provide a boundary and calculated with the permitted ±7.5% deviation, it was found that 66.1% (43 cases) of the sixty-five illicit cases fell within the acceptable range of 140 - 190 mg, allowing for errors of measurement of ±0.1 mg.

The lightest tablets were cases 90, 78 and 23, which were marked MSJ and had a mean weight between 73 – 75 mg. This corresponds with them having the smallest diameter and thickness. These tablets are not licensed for the UK market and it is unknown whether these tablets are pharmaceutically manufactured outside the UK. No legitimate tablets are available for comparison.

The lightest pharmaceutical tablets, which were manufactured by Wockhardt, had a mean weight of approximately 153.6 mg. The only illicit cases that bore the CP logo used by Wockhardt were cases 10 and 81. Case 10 contained tablets with a range from 158.5 – 161.9 mg, and a mean weight of 160.2 mg and Case 81 had a mean weight of 153.7 mg, with a range of 150.5 – 158.9 mg. Both of these cases were within the permitted ±7.5% range of deviation for weight. Case 81 had only a 0.01 mm difference in both diameter and depth from the pharmaceutical tablets and Case 10 was slightly deeper giving it a heavier weight but both tablets fell within the pharmaceutical criteria, making them consistent with the Wockhardt products in terms of physical characteristics. This suggests both cases 10 and 81 have the

potential to be pharmaceutically manufactured but diverted into the illegal supply chain.

Cases 4, 5, 14, 25, 84, 85, 131, 134, 137, 152 each had a mean weight of over 210 mg and were too heavy to be pharmaceutical tablets produced for the UK market, when compared to the data produced by the legitimate tablets tested. Interestingly, the later six cases, which had mean weights in the range of 223 – 245 mg were all marked 1/0 and were found to contain phenazepam (**16**) (Chapter 4 – GCMS). Cases 4 and 14 with the MA & D/10 markings also contained phenazepam (**16**) but had a slightly lower mean weight of approximately 220.5 mg and 213.3 mg respectively.

The only 'heavy' tablets which contained diazepam (**1**) were cases 5 and 25, which each had a mean weight between 214 – 216 mg and were both marked MA |D/10. Correspondence with MA Pharmachem indicated that their tablets should weigh between 165 - 175 mg. In total, twelve of the illicit cases with the corresponding MA | D/10 marking were found to weigh within this range (Figure 3.10). These were cases 2, 13, 16, 24, 29, 30, 74, 79, 82, 83, 86 and 159. The remaining twelve cases with the MA logo had a mean weight above this level.

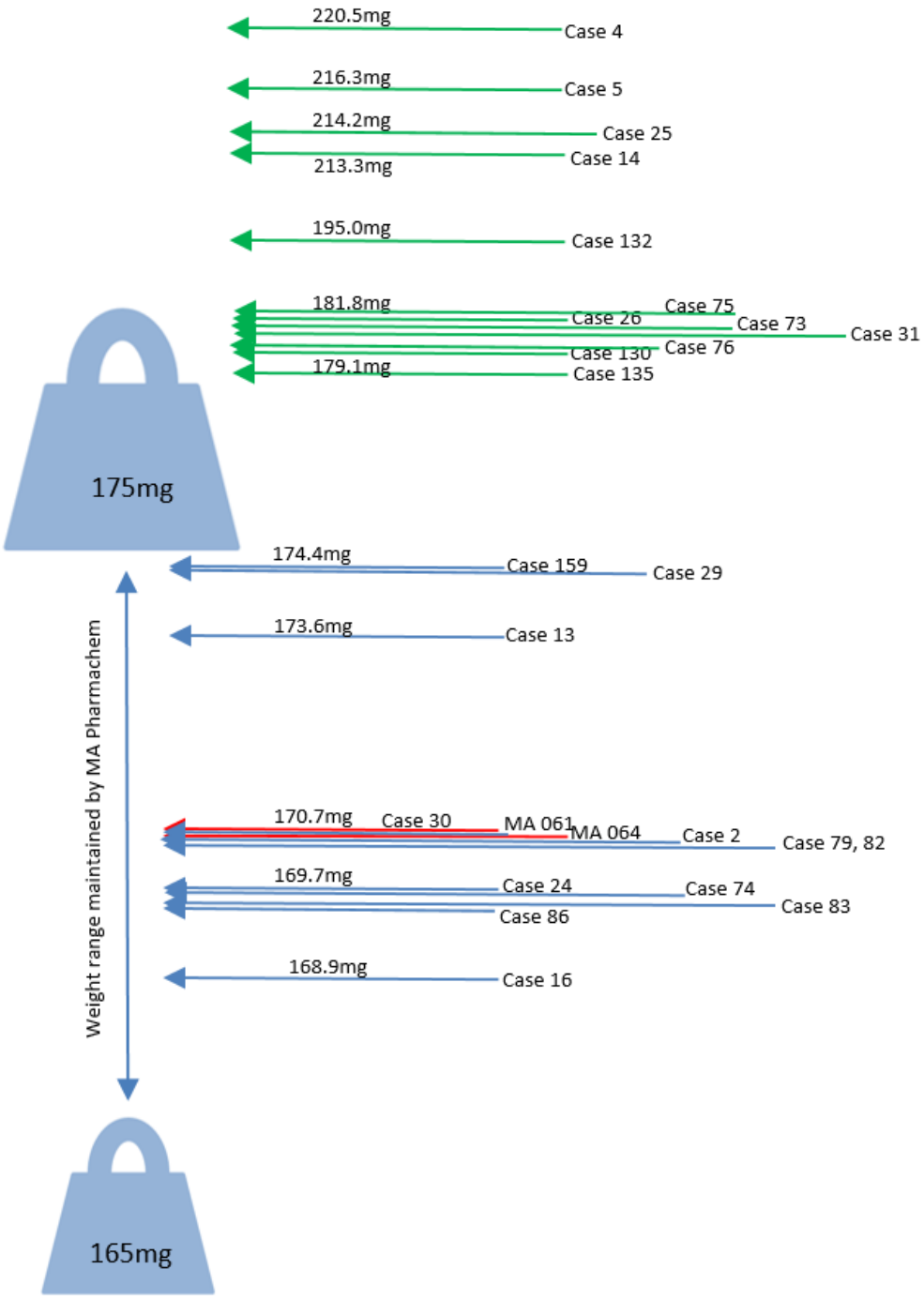


Figure 3.10. Diagram illustrating variation in mean weight of the cases marked MA | D/10 compared to the manufacturers designed range. The mean weight of the two pharmaceutical batches MA061 and MA064 were very close to each other in the centre of the weight range used by MA Pharmachem. The diagram shows that half of the twenty-four seized cases marked with the MA logo fell within the permitted range of 165 – 175 mg but twelve cases had a mean weight above the maximum 175 mg limit, even with the allowance of ±0.2 mg for errors of measurement. The diagram is not to scale.

Any comparison becomes more complicated however, due to the regulations laid down in the pharmacopoeia. The criteria states that in testing twenty units, up to two tablets can deviate in mass by up to 15% for tablets that weigh between 80 – 250 mg (British Pharmacopoeia, 2008). This would allow two of the tablets produced by MA Pharmachem, for example, to weigh between 144.5 - 195.5 mg. In reality however, manufacturers tend to be more precise than this, with the samples tested having a RSD of less than 1%.

Case 9 contained tablets with an approximate weight in the range of 179.1 – 226 mg and the largest RSD of 13.5%. As this case is imprinted with the STADA logo, it is not manufactured for the UK market. However, the high level of variation indicates little consistency in the tablet manufacture and therefore suggests that this case is unlikely to be pharmaceutically manufactured.

3.5.4 Comparison of Results

By comparing the physical data generated by the illicit cases against those of the pharmaceutical batches, the indications are that some of the cases could have the potential to have been manufactured by pharmaceutical companies and diverted into the illegal supply chain. The results of the comparison of physical data is shown in Figure 3.11 and demonstrates that only fourteen of the of the 65 seized cases demonstrated similarity to the pharmaceutical tablets in all three measurements. Cases 3 and 23 did not replicate any of the pharmaceutical characteristics.

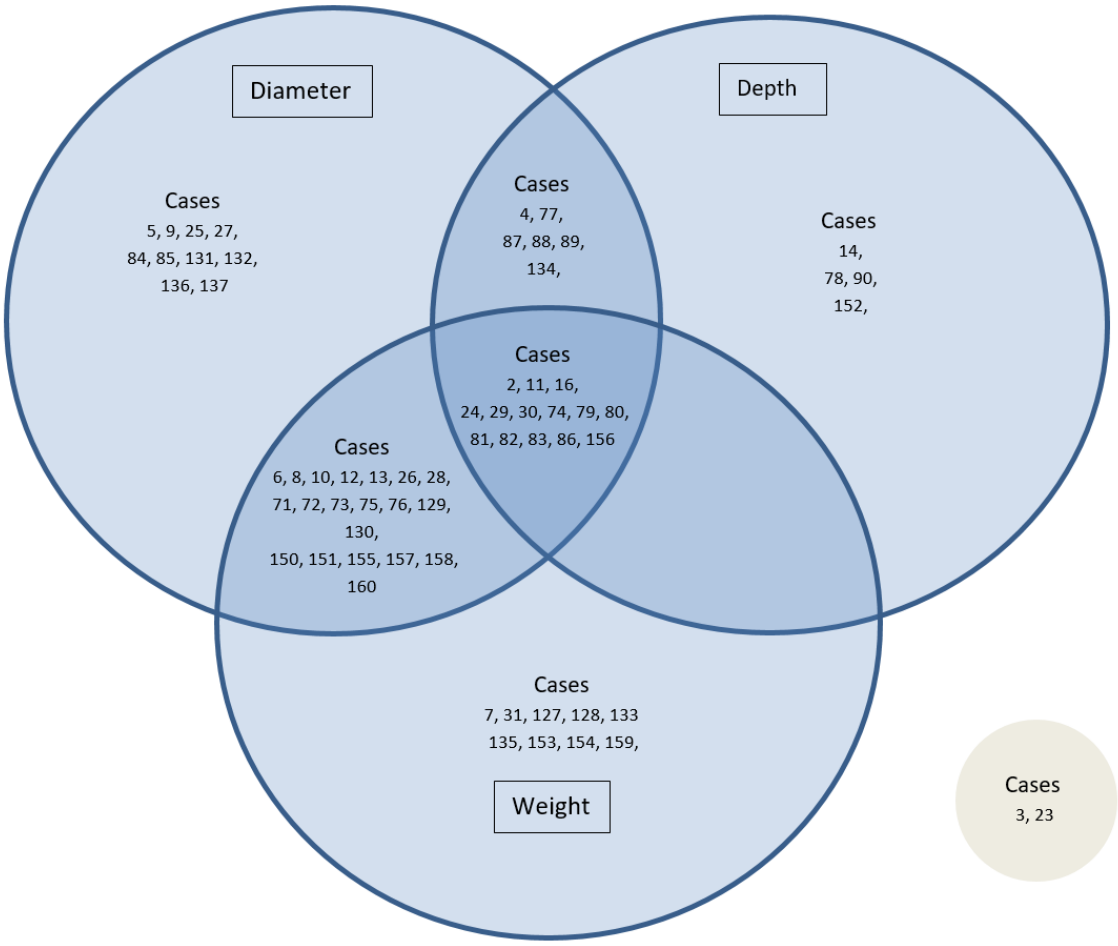


Figure 3.11. Venn diagram comparing the physical characteristics of cases against the criteria demonstrated by the known pharmaceutical tablets. Each bubble represents a different physical measurement and lists the cases which matched the pharmaceutical batch for that characteristic. Cases 3 and 23 did not comply with any of the measurements recorded for the pharmaceutical tablets.

By analysing the physical data in conjunction with the markings, it is revealed that some of the tablets have not been legitimately manufactured for the UK market. For example, Case 160 is marked EZ / 1.0 which is also not a legitimate marking for diazepam (**1**) tablets in this country and Case 12 bears the logo STADA, which is a German pharmaceutical company and does not produce diazepam (**1**) for the UK market either. Case 11 is marked Roche and the tablets seized were contained in packaging labelled as Roche, Pakistan. Roche is a Swiss pharmaceutical company that produces diazepam (**1**) tablets in other countries and notably in Asia. This case

was consistent with all of the physical characteristics demonstrated by the UK pharmaceutical tablets but is not a licensed product for the UK market.

Cases need to be compared to the pharmaceutical tablets bearing the same logo. Case 81 only weighs 153.7 mg, however it bears the logo of Wockhardt and is consistent with their pharmaceutical tablets. Case 10 also bears the Wockhardt logo but recorded a greater depth when measured, at 2.88 mm. Although this tablet fell outwith the range of depths recorded for the entire batch of Wockhardt tablets, in this study, it may be that differences between batches, or tablets produced in different factories have slight inconsistencies, such as a variation in pressure which could affect tablet depth. In addition, they may be pharmaceutically manufactured tablets that were rejected, as they met the criteria of diameter and weight. Another possibility is that tablets in Case 10 were affected by storage conditions. Therefore variation in one measurement may not entirely discount the case from being of pharmaceutical origin.

Likewise, allowing for errors of measurement, Case 133 could be described as consistent with tablets produced by Actavis. The small differences in weight and diameter of 0.07 mg and 0.12 mm respectively, above the largest and deepest tablet in the pharmaceutical batch, may be due to errors of measurement. However, it was recorded that the relative standard deviations in both depth and weight between all of the tablets in the seized case were greater, at 4.98% and 2.94%, than the pharmaceutical batch which recorded levels below 1% for each measurement.

Case 156 bearing markings consistent with tablets manufactured by Teva, was also consistent with tablets from the pharmaceutical batch. Case 80, marked Tensium, which produces legitimate diazepam (**1**) tablets for the UK market, recorded measurements consistent with all of the pharmaceutical criteria used in this study. Ten cases appeared to be consistent with MA Pharmachem tablets according to their physical measurements (cases 2, 16, 24, 29, 30, 74, 79, 82, 83, 86).

3.6 Conclusion

The results of the physical analysis of the tablets was found to be an important part of the project. The tablet markings were an integral part of the analysis and were assessed in conjunction with the physical measurements. Data generated was shown to be valuable for determining whether tablets may have been pharmaceutically manufactured, emphasising the benefits of this new research.

The analysis of physical measurements and comparison to known pharmaceutical tablets has not previously been explored with illicit blue tablets. This new research revealed both similarities and variances between different cases in the illegal supply chain and also to those of pharmaceutical origin. By comparing the tablets to pharmaceutical diazepam (**1**) tablets and exploring the data statistically, using box plots, the level of variation in measurements was highlighted giving insight into differences in consistency between pharmaceutical and illicit tablets and increasing our understanding of illicit manufacturing processes.

The findings of the physical investigation proved that some of the tablets could be identified as being of illicit origin because they were too heavy or of the wrong size. Measurements can therefore be used to differentiate between legitimate and illegitimate tablets in some of the cases. The use of box plots further revealed the amount of variation within the illicit and pharmaceutical batches and clearly indicated that many of the illicit cases contained tablets which had greater inconsistency in weight and depth. Thus indicating that a simple test into levels of variation may provide an important initial step into the origins of seized cases.

Similarities in physical characteristics, such as imprint details and diameter could also reveal potential similarities between cases, or even to pharmaceutical manufacturers. The similarities indicated that some of the seized cases may have been diverted into the illegal supply chain. Chemical analysis of the tablets, through HPLC, GC-MS and DSC later revealed that some of the potential pharmaceutical tablets contained varying levels of diazepam (**1**) or different active drug substances altogether and enabled further separation of cases begun through the physical analysis.

The comparison of the physical information brought to light potential similarities or links between some of the cases providing an important first step in differentiation between groups of tablets. The next step was therefore to ascertain chemical information regarding the illicit cases which could then be explored in conjunction with the knowledge gained by analysis of the physical characteristics. The combined results of physical and chemical tests were then analysed in further statistical tests (See Chapter 7 – Statistical Cluster Analysis).

Chapter 4. Gas Chromatography - Mass Spectrometry

4.1 Chapter Summary

Where sufficient tablet numbers allowed, Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed on at least one tablet from each of the cases in order to determine the main active drug substance present. Although the street samples were believed to be sold as 10 mg diazepam (**1**) tablets and were blue in colour, the origin of the tablets was unknown and therefore the actual content was also uncertain. In addition, any tablets containing diazepam (**1**) were not necessarily going to be of pharmaceutical origin.

The total ion chromatograms of all case samples were compared to retention time (Rt) and mass ions produced from purchased certified standards analysed using the same method conditions. Following this process allowed likely identification of the main drug substance to be made in each analysed tablet. Known pharmaceutical diazepam (**1**) standards were also run as part of the sequence, to determine any day to day drift present in Rt and on-going accuracy of the mass spectrometry detector. Standards were run both at the beginning and end of each run sequence.

4.2 Introduction

Gas Chromatography (GC) is an effective analytical technique used for separating a mixture of volatile organic compounds in a sample and when coupled with Mass Spectrometry (MS), structural information about many of the components present can be gained from the mass ions present. This technique was therefore used to provide valuable information regarding the main organic components present in the illicit blue tablets under examination. While the sensitivity of the method is determined by the limit of detection for each compound in question, in this instance, provided an active drug substance was detected in each tablet then limit of detection determination was not carried out. HPLC analysis was performed (Chapter 5) to determine the amount of main drug substance present indicated through GC-MS analysis.

The work performed in this project is important because no reports have previously been published on GC-MS analysis of illicit blue tablets with markings consistent with diazepam (**1**) tablets. This may be partly because of the large numbers of pharmaceutical tablets that were being diverted into the illegal supply chain, as described by the Advisory Council on the Misuse of Drugs (2016). However, previous work using GC-MS to confirm the identity of benzodiazepines has been performed on unknown powders and body fluids or wastewater, to investigate drug consumption (Papoutsis *et al.,* 2012; Boumba *et al.,* 2016; Fatta-Kassinos, Meric and Nikolaou, 2011).

4.2.1 Analysis of Benzodiazepines

4.2.1.1 Analysis of Impurities

GC-MS has been used widely in the pharmaceutical industry to analyse impurities (Hiriyanna and Basavaiah, 2008). The impurities in a formulated tablet include everything that is not the active drug substance, and can include precursors of synthesized substances, degradation products, reaction specific impurities and excipients (Stawny, Piekarski and Marciniec,2016).

In forensic science, impurities are analysed to identify the by-products of drug synthesis (Power *et al.,* 2013). For example, GC-MS is often used to investigate impurities found in tablets such as MDMA, because identification can give an indication of the method of synthesis, thus providing information that can potentially link cases. However, GC-MS can also identify other active drug substances present in the illicit tablets. An investigation into both the impurities and active drug content of ecstasy tablets in Hong Kong (Cheng *et al.,* 2006) revealed that although many tablets had varying quantities of MDMA, others also contained a mixture of MDMA with MDA (3,4-methylenedioxyamphetamine) and methamphetamine (MA) and seven of the 89 tablets analysed were identified as containing ketamine in levels varying between 1 – 50 mg. Such information may help link the manufacture of different batches of tablets, as it relates to formulations and manufacturing processes used. It was therefore suggested that ketamine may have been present

due to cross contamination from the production of other illicit ketamine tablets, which is widely abused in Hong Kong.

Similarly, although the primary purpose of GC-MS analysis in this project was to identify the main active drug substance present in the tablet, on occasion quantities of other active substances, such as paracetamol were identified in some of the tablets containing phenazepam (**16**). This could therefore indicate that these phenazepam (**16**) tablets form a distinct group produced by an illicit manufacturer who included paracetamol in their formulation, or as a result of contamination from other products containing paracetamol which were manufactured within the same clandestine laboratory. However, due to the low levels of active drug substance present in most benzodiazepine tablets, the levels of reaction specific impurities and degradants would be extremely low, making detection very difficult. Therefore, linking synthetic benzodiazepines based on their impurities is less practical.

4.2.1.2 Derivatisation of Benzodiazepines

Analysis should be performed on the original substance when possible in order to prevent the appearance of additional impurities and to avoid complications through incomplete derivatisation, particularly if the technique is used quantitatively. Many benzodiazepines such as diazepam (**1**), chlordiazepoxide (**3**), nordiazepam (**14**), flurazepam (**9**) and alprazolam (**2**), are volatile and do not require derivatisation (Levine, 2003) and can be analysed by GC-MS directly after extraction in methanol (M. D. Cole, 2003). One of the main excipients added to pharmaceutically produced diazepam (**1**) tablets for the UK market, is lactose (Chapter 2 Introduction, Section 2.2.4 Excipients) which is ‘very slightly soluble in alcohol’ (National Center for Biotechnology Information, 2017). Therefore, after extraction into methanol, the samples were filtered to remove any insolubles prior to filling the sample vials for analysis to prevent interference.

Derivatisation of the more polar benzodiazepines and their metabolites by silylation of active hydrogens, acylation of the polar functional groups or alkylation with amine hydrogens and acidic hydroxyl groups can lower the Rt caused through adsorption

on the stationary phase in some columns and may prevent thermal degradation of polar compounds (Levine, 2003; Kyle, 2017; Blachut *et al.,* 2004).

A ZB-5MS column was used for the analysis in this project. The 5 % - phenyl-arylene phase incorporated into a polymer of 95% dimethylpolysiloxane creates a complex network making interaction with the stationary phase more difficult and allowing preferential access based on compound shape. Therefore, the limited access causes stronger adsorption to the stationary phase, thus allowing greater resolution of structures such as multi-ring aromatic compounds, like benzodiazepines (Phenomenex, 2014).

4.2.1.3 Thermal Degradation of Benzodiazepines

A major consideration when carrying out GC-MS is the thermal stability of the compounds likely to be under investigation. Instability of some substances can result in rearrangement of the molecules and degradation. Functional groups containing active hydrogens, including the hydroxyl group (as in Oxazepam **15** which is an active metabolite of diazepam **1**) and amine group (as in Chlordiazepoxide **3**) can reduce volatility and are thermally unstable. Peak broadening may also occur due to interactions with the stationary phase (Danielson, Gallagher and Bao,2000; Lin *et al.,* 2008). As the tablets analysed in this project were illicit, the active drug substance present and its thermal stability was therefore unknown. However, chlordiazepoxide (**3**) was identified in one of the cases tested (case 136) and multiple peaks were produced due to thermal degradation.

Some benzodiazepines may be thermally unstable during GC-MS analysis. A study of sixteen benzodiazepines using capillary gas chromatography related thermal degradation directly to the chemical structure of the drugs (Joyce *et al.,* 1984). It was found that nine of the sixteen benzodiazepines tested were thermally unstable. This included ketazolam (**10**), which decomposed to diazepam (**1**) (Figure 4.1), making identification of ketazolam (**10**) and the presence of any diazepam (**1**) difficult by GC-MS alone. Therefore, for this project, the tablets identified as containing diazepam (**1**) by GC-MS were also analysed by HPLC, along with both ketazolam

(**10**) and diazepam (**1**) standards, in order to gain additional information through comparison of Rt.

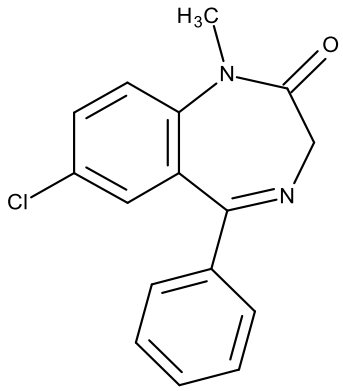
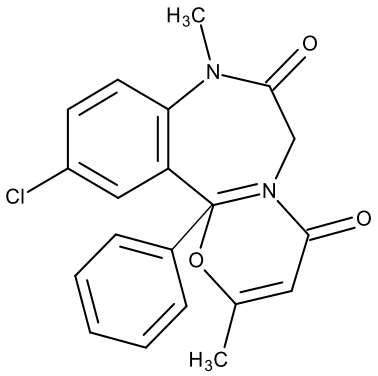
Diazepam (**1**)	Ketazolam (**10**)
	

Figure 4.1. The structure of diazepam (1) and ketazolam (10).

Chlordiazepoxide (**3**) and demoxepam (**5**) (which is an active metabolite of chlordiazepoxide (**3**) (Valentine, Middleton and Sparks, 1996)), both lose an oxygen radical due to thermal degradation in the injector port during GC (Joyce *et al.,* 1984; Blachut *et al.,* 2004). This reduces demoxepam (**5**) to the active drug substance nordiazepam (**14**), causing identification difficulties for studies where both substances may be found (Figure 4.2). Neither demoxepam (**5**) nor nordiazepam (**14**) were found in the cases analysed for this project.

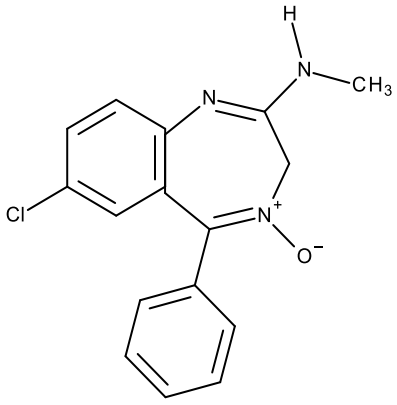
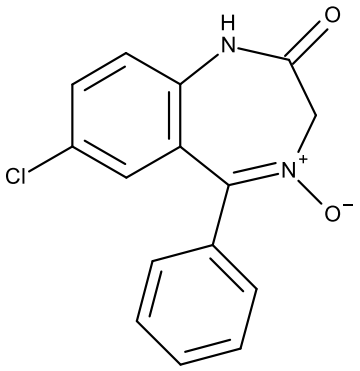
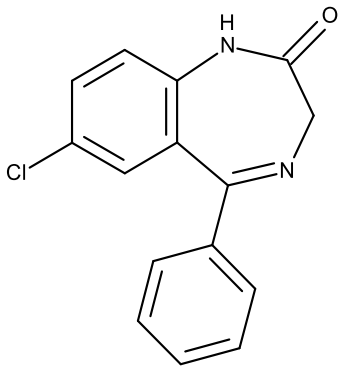
Chlordiazepoxide (**3**)	Demoxepam (**5**)	Nordiazepam (**14**)
		

Figure 4.2. The structure of chlordiazepoxide (3), demoxepam (5) and nordiazepam (14).

A report by the United Nations Office on Drugs and Crime (UNODC) (2012) noted that chlordiazepoxide (**3**), temazepam (**19**) and lormetazepam (**12**), produce multiple peaks in the GC chromatogram. Although the report notes that three peaks were detected from the chlordiazepoxide (**3**) sample, no further information regarding the resulting spectra was provided.

A study by Hida *et al (1999)*, into thermal desorption gas chromatography (TDGC) of benzodiazepines investigated differences in thermal stability of the various drug substances. The results indicated that benzodiazepines which demonstrated melting and decomposition temperatures that were considered closer together, such as chlordiazepoxide (**3**), tended to produce multiple peaks. The results of the TDGC recorded a melting temperature for chlordiazepoxide (**3**) of 242.7 ^{o}C and a decomposition temperature of 284.2 ^{o}C, resulting in seven peaks being produced. This compares to diazepam (**1**) with a greater difference in melting and decomposition temperatures of 132.3 ^{o}C and 282.9 ^{o}C respectively, which resulted in just one peak. Therefore it is important to find a balance whereby the temperature is high enough to vaporise the sample while sufficiently low to limit degradation.

Previous GC-MS analysis of benzodiazepines by Joyce *et al* (1984) and Blachut *et al* (2004) used different temperature programmes. Joyce set an injection temperature of 270 ^{o}C and the oven temperature was ramped from 50 ^{o}C at a rate of 30 ^{o}C / min to 260 ^{o}C. Blachut used an injector temperature of 250 ^{o}C and the initial oven temperature of 110 ^{o}C was maintained for 2 minutes before increasing by 15 ^{o}C / min up to 255 ^{o}C. The ramp rate was then decreased to 8 ^{o}C / min up to 300 ^{o}C and maintained for 5 minutes.

As R_t vary according to conditions and temperature, UNODC recommend that when GC-MS is used for the examination of benzodiazepines and barbiturates, all samples should be tested alongside certified standards on the same instrument and under the same conditions. (United Nations Office on Drugs and Crime, 2012). However, it is important to note that in some cases where new psychoactive drug substances have been used, certified standards may not always be available for purchase. Although this was not the case in this project, it may impact on the certainty of the drug substance identification by GC-MS alone.

4.2.2 Application of GC-MS to the Analysis of Illicit Drug Samples

A paper by Morelato *et al.* (2014) discussing how illicit drugs were examined in Australia focussed on prioritising a simple, relatively quick method of analysing MDMA seizures for intelligence purposes. The aim was to determine which methods of analysis would provide the best intelligence on links between seizures in the shortest time. The data came from 54 cases of seized tablets, divided into three groups: i) those known to be linked; ii) those suspected of being linked; and iii) those where no connection was evident. The results indicated that greater discrimination between cases was achieved through GC-MS analysis and that although using a combination of techniques provided more information, GC-MS could be prioritised in order to provide information quickly for purely intelligence purposes. However, the greater concentration of active drug substance which is often present in MDMA tablets, also results in an increase in the detection of process impurities. Consequently this is not suitable for "diazepam" (**1**) tablets, because the larger sample size required to pick up low levels of impurities may result in overloading the detector and obscuring the data actually being sought. They also studied GC-MS peak areas of the active drug substance and compared impurities. Although forty organic impurities were detected in the samples, an investigation was performed to determine whether analysis of a reduced number of the impurities could be as effective while reducing experimental time. The results indicated that analysis of all impurities was significantly more effective thus reducing the numbers could not be scientifically justified.

Due to GC-MS enabling identification of compounds in small samples, this technique was used to confirm the main active drug substance present in the illicit blue tablets analysed in this study. On occasion, the main organic excipients, such as stearic and palmitic acid, which are often found in tablets, were also detected in some of the samples. Due to the relatively small amount of active drug substance present though, the lower levels of organic impurities were likely to remain undetected. In addition, a limitation of this project is the possibility that low levels of other active drug substances have gone unrecorded. However, as the aim of the project was to compare data from a variety of chemical and physical techniques, in order to explore potential links between the illicit batches, it was believed that identification of the

main active drug substance was sufficient for this investigation. The objective was not to provide a detailed analysis of each tablet but, that the results of the GC-MS would be supported by diazepam (**1**) quantification through HPLC and thermal analysis by DSC, to further explore the excipients to reveal similarities and differences between the illicit batches. For example, although Case 137, produced a peak consistent with phenazepam (**16**), another peak with a Rt of 10.78 minutes was detected with a suggested similarity of 97.28% to caffeine, according to the National Institute of Standards and Technology (NIST) library (Number 14), however this was not verified by analysing a standard.

4.2.2.1 Mass Spectrometry

It has been noted that the presence of the halogen in benzodiazepines is particularly suited to analysis with an Electron Capture Detector (Levine, 2003; Pirnay *et al.,* 2002). Fitzgerald *et al.* (1997) chose diazepam (**1**) as the model compound to compare an ion-trap and a quadrupole mass spectrometer, using the same column and heating program. Diazepam (**1**) was selected due to its ability to elute without derivatisation, producing a good chromatographic peak shape and because of the presence of an aromatic halogen. The pure drug sample was injected in an unextracted state to avoid any interference from a matrix. The results revealed differences between the two methods which were explained by greater sensitivity of the ion-trap mass spectrometer and increased ion-ratio precision when the quadrupole mass spectrometer was in selected ion mode.

For this project, a 5977E single quadrupole mass spectrometer was used for detection. Pirnay *et al.* (2002) noted that the ion-trap detectors are not as reliable for detecting all benzodiazepines and as the active drug substance present in the illicit tablets was unknown, neither would the selected ion mode have been suitable. Therefore, the detector was used to scan the range of ions with a mass-to-charge ratio (m/z) between 40 - 600 amu.

4.3 Experimental

4.3.1 Instrumentation

The GC-MS analysis was performed on an Agilent 7820A gas chromatograph with a 5977E mass spectrometer using a ZB-5MS column with 30 mm x 0.25 mm inner diameter and 0.25 µm film thickness (Phenomenex, Macclesfield, England).

4.3.2 Method of Analysis

A syringe and 4mm filter with 0.2 µm pore size was used to remove any undissolved material before transferring the solutions into vials for analysis. The samples were injected in a split-less mode and the injection port was held at 250 ^{o}C. The carrier gas was helium, which was set at a constant flow rate of 1 mL / minute. The oven programme was set at 50 ^{o}C for 2 minutes, then ramped at 15 ^{o}C / minute to 280 ^{o}C, with the final temperature being held for 11 minutes. The ion source was maintained at 230 ^{o}C and the detector was set to acquire spectra in the range of 40 - 600 amu. The mass spectra were compared to certified drug standards, run at the beginning and end of the same run sequence and with the NIST 14 mass spectral library (NIST, Maryland, USA).

The above method for GC-MS benzodiazepine analysis method was taken from Clarke's Analysis of Drugs and Poisons and adapted for use in this project, based on information described by the Home Office Forensic Science Service based at Aldermaston (Joyce *et al.,* 1984) and on research into the application of GC-MS to the analysis of benzodiazepines (Blachut *et al.,* 2004).

The method was amended three times (Methods 2, 3 and 4) for separating etizolam (**7**) and triazolam (**18**), which were found to have similar retention times of 17.52 minutes for etizolam (**7**) and 17.59 minutes for triazolam (**18**) using the original method.

Method 2 used an initial oven temperature of 50 ^{o}C, held for 2 minutes, ramped at 15 ^{o}C / minutes to 260 ^{o}C, held for 5 minutes, then ramped at 10 ^{o}C / minutes to 280 ^{o}C and held for 10 minutes.

Method 3 had the initial oven temperature of 50 ^{o}C which was held for 2 minutes The initial ramp rate was reduced by 5 ^{o}C /minute, to 10 ^{o}C / minutes to 260 ^{o}C, which was held for 5 minutes and ramped at 10 ^{o}C / minutes to 280 ^{o}C. The temperature was held for 10 minutes.

Method 4 set the initial oven temperature at 50 ^{o}C, held for 2 minutes and ramped at 10 ^{o}C / minutes to 230 ^{o}C. The temperature was held for 30 minutes, then ramped at 10 ^{o}C / minutes to 280 ^{o}C and held for 10 minutes.

4.3.3 Materials used in this Project

HPLC grade acetonitrile, diethyl ether and HPLC grade methanol were supplied by Fisher Scientific (Loughborough, England). Chlordiazepoxide (**3**) (certified standard 1 mg / mL in methanol), diazepam (**1**) (certified standard 1 mg / mL in methanol), etizolam (**7**) (certified standard 1 mg / mL in methanol), triazolam (**18**) (certified standard 1 mg / mL in methanol), paracetamol (acetaminophen; 1 g analytical standard), aspirin, (1 g analytical standard) and bupivacaine (1 g analytical standard) were obtained from Sigma Aldrich (Gillingham, England). Ketazolam (**10**) (certified standard 1 mg / mL in methanol) and phenazepam (**16**) (certified standard 1 mg / mL in methanol) were purchased from LGC (Middlesex, England). MA tablets were supplied by MA Pharmachem Ltd (Bolton, UK); Wockhardt tablets were supplied by Wockhardt UK Ltd (Wrexham, UK); Teva tablets were supplied by Teva UK Ltd (Runcorn, UK); Actavis Tablets were supplied by Actavis Ltd (Barnstaple, UK); and illicit blue tablets were obtained from Police Scotland.

4.3.4 Preparation of Standards

Standards of diazepam (**1**), ketazolam (**10**), phenazepam (**16**), etizolam (**7**), triazolam (**18**), chlordiazepoxide (**3**), promethazine, aspirin and paracetamol were prepared separately, from certified drug standards diluted with methanol to produce a working stock solution of 0.1 mg / mL.

4ml of each 0.1mg / mL drug standard was pipetted into a separate 5ml flask. 1mL of an internal standard of 0.1mg /mL bupivicaine solution used to make up to the mark. Resulting in a flask containing 0.08 mg / mL drug standard and 0.02 mg / mL bupivacaine.

Standards were analysed both individually and in combination with other drug standards. Vials were prepared for analysis containing an equal combination of diazepam (**1**), phenazepam (**16**), etizolam (**7**), triazolam (**18**) and bupivacaine all taken from working stock solutions with the concentration of 0.1 mg / mL. Resulting in a solution containing 0.02 mg / mL of each drug substance.

Additional vials were prepared with a combination of triazolam (**18**), etizolam (**7**) and the bupivacaine internal standard by adding equal amounts of each 0.1 mg / mL working stock solution resulting in 0.033 mg / mL of each drug in the final solution.

Vials were also prepared containing equal amounts of the 0.1 mg / mL phenazepam (**16**), aspirin, paracetamol and bupivacaine standards, resulting in a solution containing 0.025 mg / mL of each drug in the final solution.

The certified standards were run at the start and end of each sample sequence

4.3.5 Preparation of Samples

4.3.5.1 Preparation of Samples using Diethyl Ether

A single tablet from each case was quartered and one quarter of the tablet was taken for analysis (or one eighth for cases indicated as containing over 20 mg of diazepam (**1**) by HPLC analysis). The remaining three-quarters of the tablet was retained for future experimentation.

The one quarter tablet was crushed in a folded piece of filter paper. The powdered sample was added to a test tube containing dilute ammonia (2 M; 1 mL) and the test tube was gently agitated to dissolve the tablet. Diethyl ether (2 mL) was added to the test tube and it was gently agitated to facilitate the extraction of the sample into the diethyl ether. A sample was taken from the upper diethyl ether layer and transferred to a GC-MS vial using a Pasteur pipette prior to analysis.

4.3.5.2 Preparation of Samples using Methanol

A quarter (or eighth for cases containing over 20 mg of diazepam (**1**) by HPLC analysis) of a tablet was crushed in a folded piece of filter paper. The powdered sample was added to methanol (1 mL) a test tube. The test tube was gently agitated to dissolve the drug substance. A syringe and 4mm filter with 0.2 µm pore size was then used to filter any undissolved material before transferring the solutions into vials for analysis.

4.3.5.3 Preparation of whole tablets using Methanol

A whole tablet from cases where no chromatographic peaks were found from smaller samples (quarter tablets, using both methanol and diethyl ether extraction, then half tablets), was individually crushed in a piece of folded filter paper and added to a test tube. Methanol (2 mL) was added and the solution was agitated to dissolve the drug substance and the sample was filtered into a GC-MS vial.

4.3.6 Overview of the GC-MS analysis performed

4.3.6.1 Benzodiazepines in Salt and Freebase Form

According to a report by the United Nations Office on Drugs and Crime (2012), the majority of benzodiazepines encountered are in the free base form and likely to be soluble in methanol alone, or on occasion, they may be in the form of a hydrochloride salt or mesylate. For qualitative analysis, the report states that most benzodiazepines are soluble in methanol, whether in free base / acid or salt form and this was the recommended method for qualitative analysis (United Nations Office on Drugs and Crime, 2012).

The tablets analysed in this study were illicit and therefore neither the active drug substance present, nor their form, was known. Therefore, the drug substances present in the illicit tablets were converted to their freebase form by adding dilute ammonia and then extracted into diethyl ether. In addition, the illicit tablets were also subjected to GC-MS after only being dissolved in methanol.

4.3.6.2 Sample size used for GC-MS Analysis

GC-MS is a sensitive technique and therefore a quarter or an eighth of a tablet was initially used for analysis. This was to allow multiple experiments to be performed, including initial drug identification by GC-MS, followed by a further run for comparison alongside the certified standard; thus leaving the remaining portion for DSC. Additional tablets from cases containing diazepam (**1**) were further analysed by HPLC and these Rt compared with that of certified diazepam (**1**) and ketazolam (**10**) standards as an additional consistency check. However, cases containing etizolam (**7**) or phenazepam (**16**), such as cases 76 and 77, did not produce an identifiable chromatographic peak on the GC-MS, when analysing a quarter tablet, therefore the sample size was increased to a half tablet and then a whole tablet, as required.

With the exception of the early cases, where only one or two tablets were available for analysis. For cases where the active substance was not detected, the analysis was repeated another two times, in case of inhomogeneity. When the active

substance remained undetected, a half tablet was analysed and then, when unsuccessful, a whole tablet, when tablets numbers allowed. As little difference was found in the results but more identifiable peaks were produced using methanol, this was the method used for the whole tablet preparations.

4.3.6.3 Run sequence of Samples

Each run sequence of cases began with a blank to ensure there was no carry over from previous runs. The blank was a vial of methanol when the tablet preparation used methanol and a blank of diethyl ether when the preparation method used dilute ammonia and diethyl ether.

The second vial in a sequence was a diazepam (**1**) standard in order to provide a comparison and Rt for the sequence. This was followed by a blank in order to pick up any carry over. In cases of carry over, which occurred on the initial analysis of case 136 and the certified chlordiazepoxide (**3**) standard, the run time of the sequence was lengthened.

Samples prepared from the illicit cases were then run, alternating with blanks until the end of the sequence. Finally, a second diazepam (**1**) standard was run followed by a blank to complete the sequence. The second standard ensured consistency of the results and monitored drift. The Rt and spectra were compared between the standards and the case samples to corroborate initial results suggested from the mass spectral libraries to determine whether the certified standard did indeed produce the same mass ions and Rt as the case samples. Only then, was the identification accepted.

Diazepam (**1**) standards were run at the beginning and end of every sequence. However, when samples did not have Rt or mass spectra which corresponded to the diazepam (**1**) standard, a comparison was made to the NIST 14 library, to give a tentative identification. The sample was then rerun with a vial of mixed standards and a separate standard of the active substance suggested by the NIST 14 library, in order to confirm similarities in Rt and mass ions using the same instrumentation and methodology.

This method of analysis allowed a direct comparison of Rt and the mass ions to be performed, for each of the illicit cases. The aim of the analysis was to provide an identification of the main active drug substance present within each of the cases by comparing results of the samples to certified standards and the NIST 14 library. As drug substances were identified in all case samples and no quantification was attempted using GC-MS, then, the limit of detection (LOD) and limit of quantification (LOQ) for the GC-MS methodology were not carried out during this project.

4.3.6.4 Small Case Size

Some of the illicit cases analysed for this project contained only a small quantity of tablets. The aim of the project was to characterise cases of illicit blue tablets and build statistical models with the potential to differentiate between different groupings. Since police seizures vary in size having valid statistical models that are appropriate for small case numbers may be beneficial. However, this does mean at times that the analysis performed has to be prioritised in order to gain the most useful information. Initial screening results carried out by the Robert Gordon University suggested that cases 7 and 10 contained diazepam (**1**). Although not enough tablets were available for GC-MS analysis, samples from these two cases were however, run alongside a diazepam (**1**) standard on the HPLC for quantification purposes and remaining fragments were thermally analysed using differential scanning calorimetry (DSC) in order to gain information about excipients.. In both of these cases, the single chromatographic peak produced by the HPLC analysis corresponded with the Rt of the diazepam (**1**) standard. The thermograms resulting from DSC indicated that their thermal profiles were consistent with the presence of diazepam (**1**). Therefore, an assumption has been made that both cases 7 and 10 contained diazepam (**1**).

Similarly, cases 4 and 14 were found to contain phenazepam (**16**) in screening tests performed at Robert Gordon University. Again, HPLC analysis indicated that the single chromatographic peak produced by samples from each of the two cases corresponded to the Rt produced by the certified phenazepam (**16**) standard and DSC thermograms were also consistent with phenazepam (**16**). Therefore, the assumption was made that both cases 4 and 14 contained phenazepam (**16**).

4.4 Results and Discussion

The method used was intended to be a balance between those used in two previous studies. The research by Blachut (2004) and Joyce (1984) both revealed thermal degradation of the benzodiazepines, including complete degradation of ketazolam (**10**) to diazepam (**1**) and decomposition of chlordiazepoxide (**3**) (See section 4.2.1.3. Thermal Degradation). This may have been due to the higher injection temperature set by Joyce and high oven temperature used by Blachut. The similarity of melting and degradation temperatures as suggested by Hida (1999) indicated that lower temperatures may limit some of the degradation. Therefore, for this project, an injection temperature of 250 ^{o}C was used, the same as in Blachut's research (2004) but the lower oven temperature (from Joyce (1984)) was selected. It was ramped at 15 ^{o}C / min (the same as in Blachut's study) but to 280 ^{o}C, a temperature between the intermediate and higher levels previously used by Blachut. Both research studies had used a split-less injection for the samples. Both split and split-less injections were tested in this project, with no significant difference in the results, other than the smaller peaks becoming more difficult to detect.

Analysis of individual standards was carried out in order to determine the relative Rt, resolution and mass ions of the different active drug substances using the methodology in this study and an internal standard of bupivacaine was used to monitor relative Rt for drug substances. Additional vials containing a combination of triazolam (**18**), etizolam (**7**) and the bupivacaine certified standards were also analysed as part of the run sequence comprising of the unknown samples with Rt of around 17 minutes. This was performed as an added comparison because of the similarity in Rt between the triazolam (**18**) and etizolam (**7**) standards and because etizolam (**7**) was not identified using the NIST 14 library. Identification was facilitated by their respective mass ion fragmentation patterns.

Vials were also prepared containing equal amounts of phenazepam (**16**), aspirin, paracetamol and bupivacaine standards as a comparison to samples which produced peaks with a match of over 90% to phenazepam (**16**), paracetamol and aspirin according to the NIST 14 library.

The certified standards were run at the start and end of each sample sequence to demonstrate that there had been no significant drift in Rt during the run. The standards produced distinct chromatographic peaks with mass ion fragmentation patterns that corresponded well with the instrument mass spectral libraries. This gave confidence that the method was capable identifying the active drug substances. The results produced by the combined standards can be seen in Figure 4.3.

Differentiation between ketazolam (**10**) and diazepam (**1**) was supported by HPLC analysis as the certified standards were found to produce peaks with different Rt, whereas GC-MS alone, may result in ketazolam (**10**) undergoing thermal degradation to diazepam (**1**) as detailed in paragraph 4.2.1.3 ‘Thermal degradation’ (above) and section 4.4.1 ‘Diazepam and Ketazolam’.

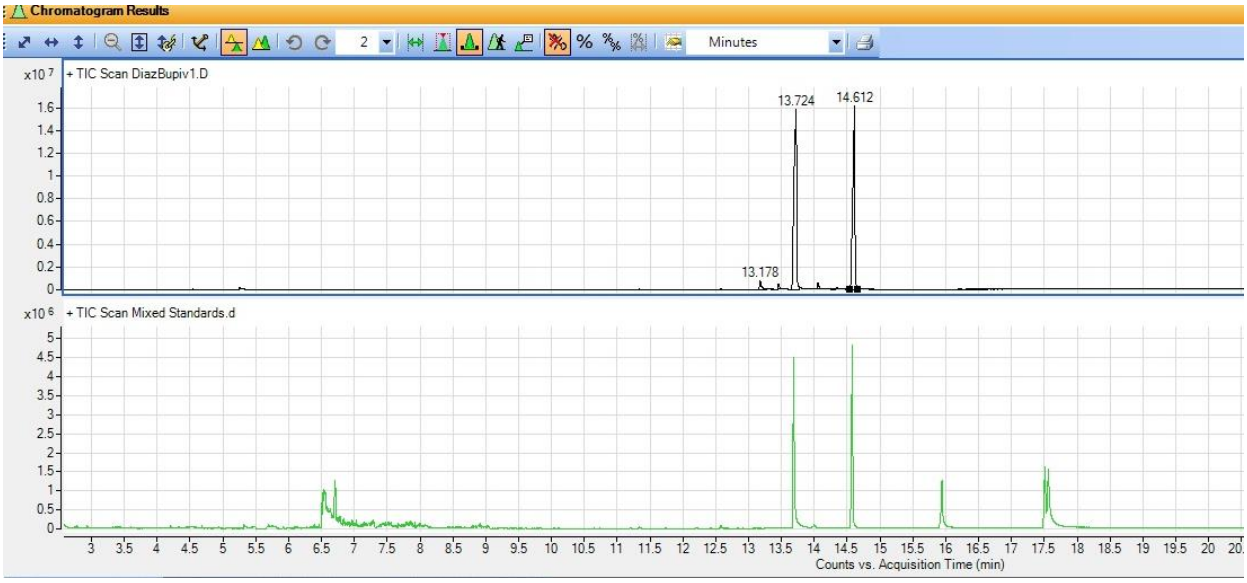


Figure 4.3. Chromatogram of the diazepam (1) standard compared to a sample of mixed standards. The upper image is the diazepam (1) standard, and shows the two peaks produced by the bupivacaine enantiomers at 13.17 and 13.72 minutes. The bottom image shows the chromatogram produced by the mixed standards. The small broad peak at about 6.5 minutes was not identified. The remaining peaks are from left to right, bupivacaine (R_t 13.72 minutes), diazepam (1) (R_t 14.61 minutes), phenazepam (16) (R_t 15.95 minutes) and the unresolved peak of etizolam (7) and triazolam (18) (17.52 and 17.59 minutes).

4.4.1 Diazepam and Ketazolam

Using the instrumentation and methodology stated above, analysis of the certified diazepam (**1**) standard, demonstrated a 97.78% similarity to the NIST 14 library. Comparison of the spectra produced by the standard revealed that the eight ions with greatest abundance (m/z 256, 283, 221,165, 110, 77, 89, 177) were consistent with the diazepam (**1**) spectrum recorded on the NIST 14 library. However, there was a difference in the order of abundance between m/z 165 and 110. The certified standard also demonstrated similar abundance between mass ions at 177 and 241, therefore contributing to the slight discrepancy in similarity. These discrepancies were likely to be a result of having different MS conditions on different machines.

The diazepam (**1**) spectra produced by the pharmaceutical tablets was consistent with the certified standard, applying the methodology and equipment used in this

analysis. The exceptions were the similar abundance of the mass ions 165 and 110 and that the analysis of the pharmaceutical tablet identified a greater abundance of m/z 241 than 177, thus resulting in a similarity of 97.46%. The differences in abundance of the first nine mass ions are shown in Table 4.1.

Table 4.1 Comparison of mass ion abundance between the NIST 14 library, a certified diazepam (1) standard and a known pharmaceutical tablet produced by MA Pharmachem.

Diazepam Mass Ion	NIST 14 Library Abundance (%)	Certified Standard Abundance (%)	Pharmaceutical Tablet (MA) Abundance (%)
256	29.24	31.75	32.89
283	28.07	30.16	31.25
221	11.11	9.52	9.87
165	5.85	5.40	5.26
110	5.56	6.03	5.26
89	5.56	4.76	3.95
77	5.56	4.76	3.95
177	5.26	3.81	3.62
241	3.80	3.81	3.95

Using the methodology described above, the certified diazepam (**1**) standard recorded a Rt of 14.57 minutes on the first analysis. However, there was a slight drift over the course of the analysis between 14.55 – 14.62 minutes. Analysis of the pharmaceutical tablet manufactured by MA Pharmachem demonstrated a Rt and mass ions similar to the certified standard, therefore demonstrating consistency with the certified drug substance using this methodology.

4.4.1.1 Detection of Ketazolam (10)

During the initial runs of the illicit tablets, the suggested identification from the mass ion fragmentation pattern and the NIST 14 library for three of the illicit cases (Cases 150, 156 and 159) was ketazolam (**10**). Therefore, a certified standard of ketazolam (**10**) was obtained and run alongside the diazepam (**1**) standard and the relevant cases for comparative purposes. The ketazolam (**10**) standard was found to produce a chromatographic peak at the same Rt as diazepam (**1**) at 14.61 minutes (Figure 4.4) along with matching spectra (Figure 4.5). In addition, no molecular ion was produced by the certified ketazolam (**10**) standard, during this analysis, supporting the complete degradation to diazepam (**1**) recorded by Joyce *et al.*

(1984). This indicated that the ketazolam (**10**) molecule fragmented to form the same ions as the diazepam (**1**) standard, thus making a definitive identification by GC-MS very problematic. The chromatographic peaks at 13.17 and 13.72 minutes relate to enantiomers of the internal standard of bupivacaine, used to monitor drift.

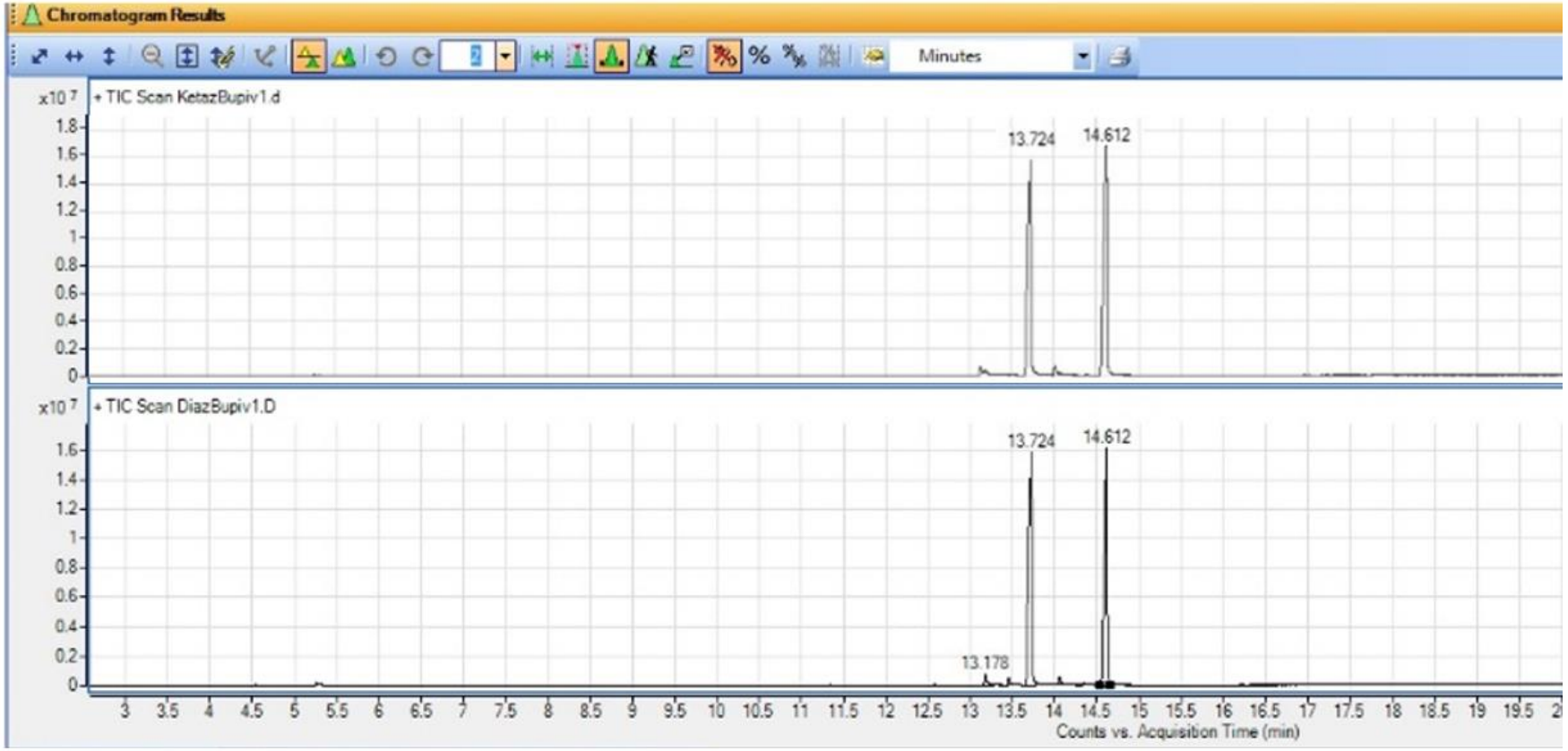


Figure 4.4. Comparison of GC-MS chromatogram of the certified ketazolam (10) and diazepam (1) standards. The peaks at 13.17 and 13.72 minutes, relate to the enantiomers from the internal Bupivacaine standard.

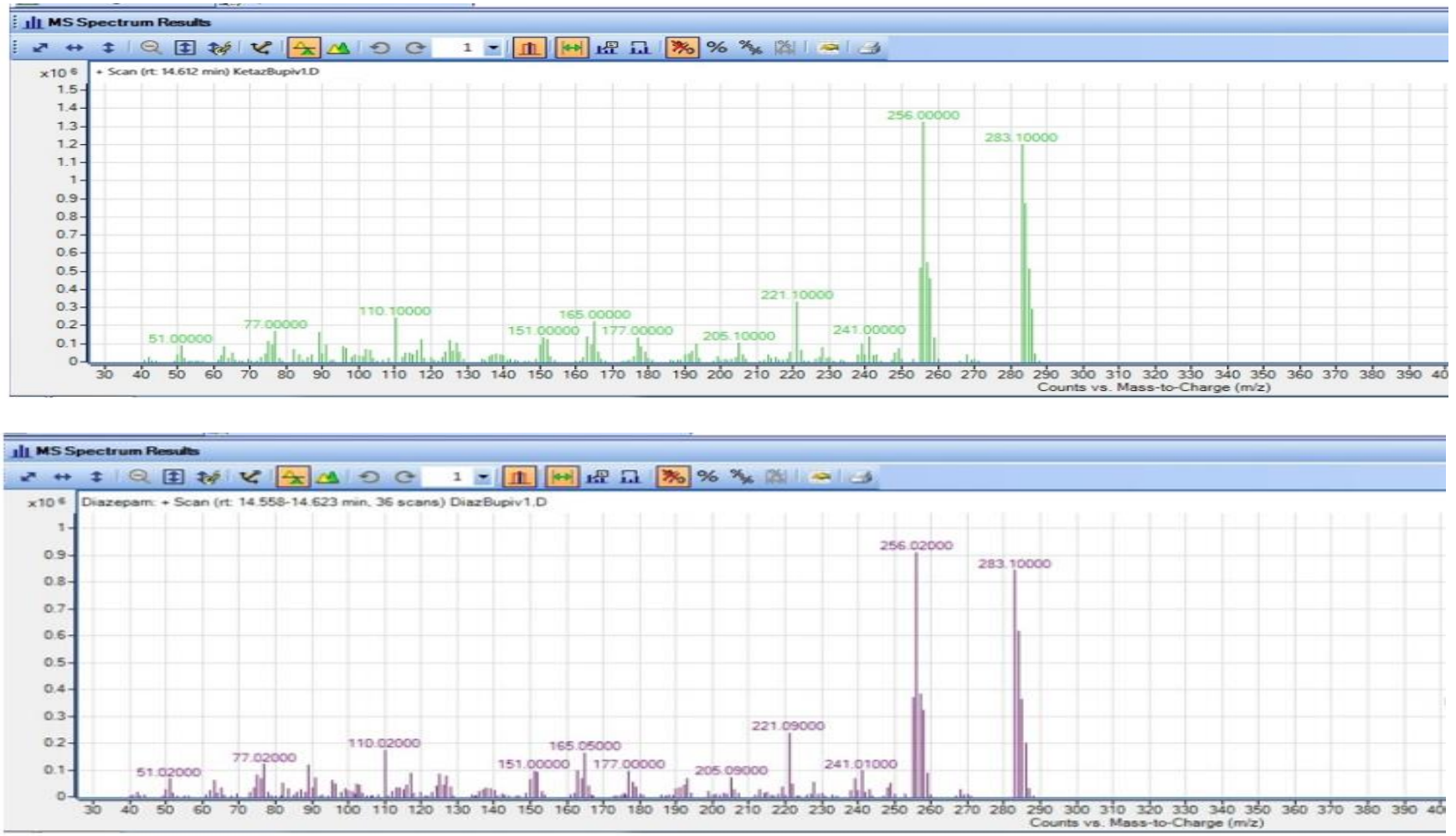


Figure 4.5. Comparison of GC-MS spectra of the certified ketazolam (10) and diazepam (1) standards.

As discussed in Section '4.2.1.3. Thermal Degradation', it is likely that due to its thermal instability, ketazolam (**10**) degrades by losing $C_4H_4O_2$. This thermal degradation to diazepam (**1**) is indicated below in Figure 4.6.

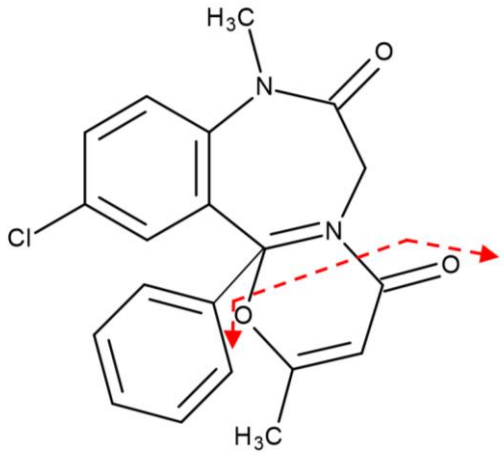


Figure 4.6. The structure of ketazolam (10). Thermal instability causes ketazolam (10) to degrade to diazepam (1) during gas chromatography unless the sample has been derivatised (Joyce *et al.,* 1984)

While the difference between the two drug substances could not be easily distinguished by GC-MS alone without carrying out a derivatisation process, it was possible to separate both the standards and illicit tablets successfully by high performance liquid chromatography (HPLC) as discussed in Chapter 5, where both diazepam (**1**) and ketazolam (**10**) were found to have good chromatographic separation, with Rt of 8.8 minutes and 11.4 minutes respectively.

Using the methods described above, the certified diazepam (**1**) standard generated a peak with the Rt of 14.61 minutes and ions with mass to charge ratio (m/z) of (in order of abundance) 256, 283, 221, 165, 110, 77, 241, 205, 51, 151 and 177 (Table 4.2). This was supported by the identification indicated by the NIST 14 mass spectral library which listed the mass ions in the following order of abundance 256, 283, 221, 165, 110, 77, 89, 177 with a suggested similarity of 97.78%.

The certified diazepam (**1**) standard was run at the beginning and end of every sequence in order to provide confidence in the results. Peaks with the same Rt and ions were identified in all of the pharmaceutical tablets and the illicit cases listed in Table 4.3.

Table 4.2 Table showing the Rt and m/z of the eight most abundant ions for the certified drugs standards analysed.

Certified Drug Standard	NIST m/z abundance (%)	Retention Time	m/z (In order of abundance %)
Diazepam (1) (NIST 97.78%)	256(31.8%), 283(30.2%), 221(9.5%), 110(6.0%), 165(5.4%), 77(4.8%), 89(4.8%), 241(3.8%)	14.61	256(31.8%), 283(30.2%), 221(9.5%), 110(6.0%), 165(5.4%), 77(4.8%), 89(4.8%), 241(3.8%)
Phenazepam (16) (NIST 98.65%)	321(24.8%), 350(17.6%), 75(13.1%), 177(10.6%), 89(9.2%), 103(8.9%), 151(7.9%), 205(7.9%)	15.95	321(27.5%), 350(22.8%), 103(13.7%), 75(9.9%), 177(9.9%), 151(6.9%), 205(5.2%), 285(4.1%)
Etizolam (7)		17.52	342(44.0%), 313(15.0%), 266(13.2%), 224(10.6%), 137(6.2%), 75(4.4%), 102(3.5%), 45(3.1%),
Triazolam (18) (NIST 93.14%)	313(22.9%), 238(15.6%), 315(15.6%), 342(11.5%), 75(10.3%), 137(8.7%), 203(8.0%), 102(7.4%)	17.59	313(25.4%), 342(23.9%), 238(16.0%), 75(8.1%), 137(7.4%), 203(6.6%), 102(6.3%), 279(6.3%)
Ketazolam (10) (NIST 86.12%) Diazepam (1) (NIST 96.18%)	256(34.6%), 283(24.2%), 110(8.3%), 221(8.0%), 84(6.9%), 69(6.6%), 325(5.9%), 165(5.5%)	14.61	256(34.6%), 283(31.1%), 221(9.0%), 110(6.6%), 165(5.9%), 77(4.5%), 89(4.5%), 241(3.8%)
Chlordiazepoxide (3) (NIST 63.54%)	282(40.0%), 299(15.2%), 77(8.8%), 56(8.0%), 91(7.6%), 241(7.2%), 44(6.8%), 220(6.4%)	14.83	270(49.7%), 219(11.9%), 313(9.4%), 77(8.5%), 239(7.0%), 44(6.0%), 207(4.0%), 165(3.5%)
(NIST 91.28%)		15.02	282(67.2%), 220(10.7%), 163(4.0%), 124(4.0%), 247(4.0%), 77(4.0%), 190(3.4%), 51(2.7%)
(NIST 96.63%)		16.67	282(54.5%), 220(8.5%), 247(6.8%), 77(6.8%), 163(6.2%), 123(5.6%), 205(5.1%), 44(4.5%)
Promethazine (NIST 95%)	72(78.7%), 180(5.5%), 284(3.1%), 198(3.1%), 213(2.4%), 152(2.4%), 56(2.4%), 44(2.4%)	17.69	72(82.6%), 284(5.0%), 180(5.0%), 198(3.3%), 44(2.5%), 154(1.6%)

A tablet taken from the batch manufactured by MA Pharmachem was analysed by GC-MS in order to provide a comparison for illicit cases marked MA / D10 containing diazepam (**1**). The MA / D10 logo was the most common marking found on the seized tablets, with twenty-four out of the sixty-five illicit cases bearing these markings.

The known MA pharmaceutical tablet produced a chromatographic peak which corresponded with diazepam (**1**) and two further peaks with Rt of 11.66 and 12.89 minutes, which were identified as palmitic acid (n-hexadecanoic), with a match of 97.76% in the NIST 14 library and stearic acid (octadecanoic) with a match in the NIST 14 library of 95.97%, respectively. However, no further analysis was carried out in attempt to confirm the identity of the palmitic and stearic acid in this project but the importance of these compounds is further discussed in section 4.4.5 'Lubricants'.

Analysis of the twenty-four illicit cases marked MA, indicated that six contained active ingredients other than diazepam (**1**). Eight of the cases were later shown by HPLC analysis to contain levels of diazepam (**1**) which varied significantly from the anticipated 10 mg amount present in pharmaceutical tablets. Interestingly, the remaining ten illicit cases produced a GC-MS chromatogram which visually matched that generated by the MA pharmaceutical tablets, with the three peaks assigned to diazepam (**1**), palmitic and stearic acid and similarity in the mass spectra. These ten cases (2, 16, 24, 29, 30, 74, 79, 82, 83 and 86) were also found by HPLC analysis, to contain 10 mg of diazepam (**1**). Thus when information on physical characteristics was examined in conjunction with both chromatographic and mass spectrum data, tablets from each of these ten cases were found to be consistent with pharmaceutical tablets diverted into the illegal supply chain.

4.4.2 Identification of Phenazepam

Using the methods described above, the certified phenazepam (**16**) standard generated a peak with the Rt of 15.95 minutes and the ions as shown in Table 4.2. This was supported by the identification indicated by the NIST 14 mass spectral library with a similarity of 98.65%.

All cases which had an initial indication of phenazepam (**16**) according to the NIST 14 library, were re-tested with a certified phenazepam (**16**) standard run at the beginning and end of all sequences. All of the cases with the initial indication of phenazepam (**16**) produced peaks with the same Rt and ions as the phenazepam (**16**) standard. Table 4.3 gives a brief summary of the main active drug substances found within the illicit cases.

Table 4.3. Table identifying the active ingredients detected in the illicit cases by GC-MS.

Substances Detected	Case Number
Diazepam (1)	2, 3, 5, 6, 8, 9, 11, 12, 13, 16, 23, 24, 25, 26, 27, 28, 29, 30, 31, 71, 72, 74, 75, 78, 79, 80, 81, 82, 83, 86, 90, 129, 130, 133, 150, 156, 159
Phenazepam (16) + Paracetamol and aspirin + Caffeine	77, 84, 85, 87, 88, 89, 131 134, 152 137
Etizolam + Aspirin + Chlorphenamine	76, 132, 135, 158 73 151, 155, 157, 160
Chlordiazepoxide (3) (+ Diazepam – maybe a product of degradation)	 136
Promethazine	127, 128, 153, 154

In addition to a peak resembling phenazepam (**16)** with a Rt of 15.95 mins and similar mass ions produced, Cases 134 and 152 were also found to contain both a large peak consistent in Rt and mass ions with the paracetamol standard and another with the aspirin standard (Figures 4.7 and 4.8). These were first identified in the initial GC-MS run and then confirmed by rerunning the illicit tablets alongside standards both by GC-MS and HPLC.

Similarly, case 137 produced a peak consistent with phenazepam (**16**) but a peak with the Rt of 10.78 minutes was also detected. This had a suggested match of 97.28% to caffeine, according to the NIST 14 library but it was not verified by analysing a certified standard. The presence of caffeine was not detected in other cases containing phenazepam (**16**), therefore distinguishing it apart from the other illicit cases. This was supported by DSC analysis, which produced a thermal profile which was distinct from other cases identified as containing phenazepam (**16**). This

is likely to have been because phenazepam (**16**) and caffeine have similar melting points of 225-230 ^{o}C and 235^{o}C respectively and because caffeine sublimes at 178 ^{o}C, which would have had an impact on thermal analysis (See Chapter 6 – Differential Scanning Calorimetry).

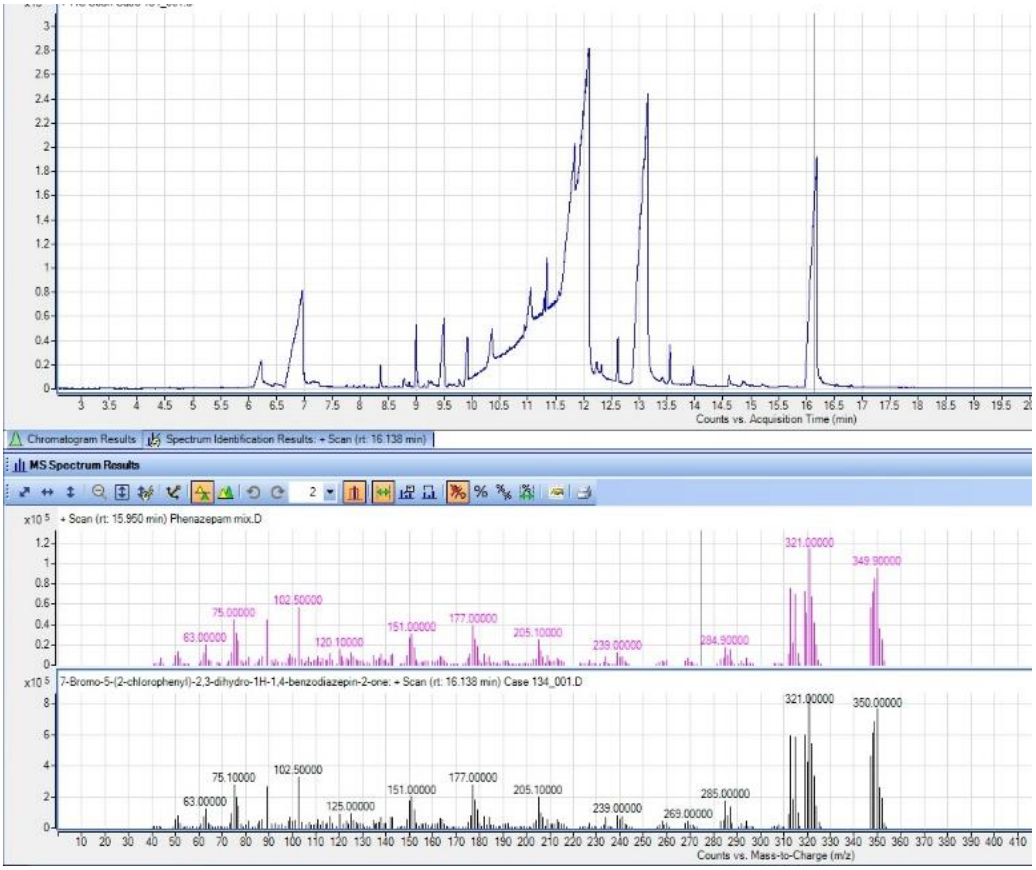


Figure 4.7. Spectra of Case 134. The peaks at 6.28 and 6.97 minutes were identified as aspirin and the broad peak from 10-12 minutes was identified as paracetamol and metacetamol. The peaks at 13.00 minutes and 15.95 minutes indicated stearic acid and phenazepam (16) respectively. The peak at about 15.95 minutes corresponds to the phenazepam (16) of the mixed standard (bottom image).

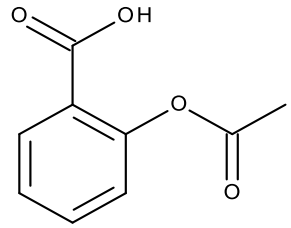
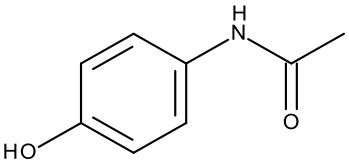
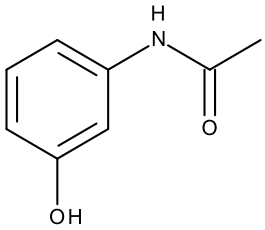
Aspirin	Paracetamol	Metacetamol
		

Figure 4.8. Structures of aspirin, paracetamol and metacetamol.

4.4.3 Chromatographic Separation of Etizolam and Triazolam

GC-MS results revealed that etizolam (**7**) and triazolam (**18**) had a very similar Rt at less than 30 seconds apart (Figure 4.9) at 17.52 and 17.59 minutes respectively. Although the peaks were distinct, the resolution was not satisfactory. In order to further resolve the chromatographic peaks, adjustments were made to the oven temperature and ramp rate, as follows:

The original method had an initial oven temperature at 50 ^{o}C, hold for 2 minutes, ramp at 15 ^{o}C / minutes to 280 ^{o}C, hold for 11 minutes. The amended method 2 introduced an intermediate step by separating the ramp in temperature into two stages, with the oven held 260 ^{o}C for 5 minutes, before ramping at 10 ^{o}C / minutes to 280 ^{o}C and holding for 10 minutes. The results indicated that slowing the ramp rate by adding an intermediate stage did not resolve the peaks but delayed them from the original Rt of 17.52 minutes for etizolam (**7**) and triazolam (**18**) at 17.59 minutes to 18.18 minutes and 18.32 minutes respectively. Maintaining temperature can help with the separation of substances with similar Rt (Agilent Technologies, 2007) however, despite the temperature being held for 5 minutes before the final ramp, the separation was not significant enough to resolve the peaks.

Method 3 was used in order to benefit from differences in vaporisation temperature between etizolam (**7**) and triazolam (**18**), by reducing the ramp rate to 10 ^{o}C /minute. However, the change in temperature resulted in delaying the elution, with etizolam

(**7**) having a Rt of 24.22 minutes and triazolam (**18**) eluting at 24.57 minutes with no further significant resolution.

By holding the temperature for 5 minutes, as an intermediate stage, in method 2, a small increase in the separation between the two peaks had been produced but had not achieved complete resolution. Therefore, method 4 was introduced to hold the temperature for 30 minutes, to test whether the separation could be enhanced. Complete resolution was still not achieved but broader peaks were produced with a Rt of 29.50 minutes for etizolam (**7**) and 29.93 minutes for triazolam (**18**).

The change in methodology did not result in the complete resolution of etizolam (**7**) and triazolam (**18**) but increased the run time to 65 minutes. This new methodology also increased the Rt of other benzodiazepines included in the mixed standards run along with the illicit cases, while also producing broader peaks.

In addition, etizolam (**7**) was not listed in the NIST 14 library and was therefore labelled as 'not identified'. In order to resolve these issues, both etizolam (**7**) and triazolam (**18**) standards were analysed before and after tablets suspected of containing either drug in order to directly compare Rt as well as the mass spectra. It was noted that while the Rt were similar, the spectra produced for each drug standard were notably different (Figure 4.10) thereby making identification possible, based on the certified standard used for the analysis. The Rt of the remaining standards used in the analysis were distinct and fully resolved isolated peaks were produced. Comparison of the Rt and spectra therefore enabled identification to be made.

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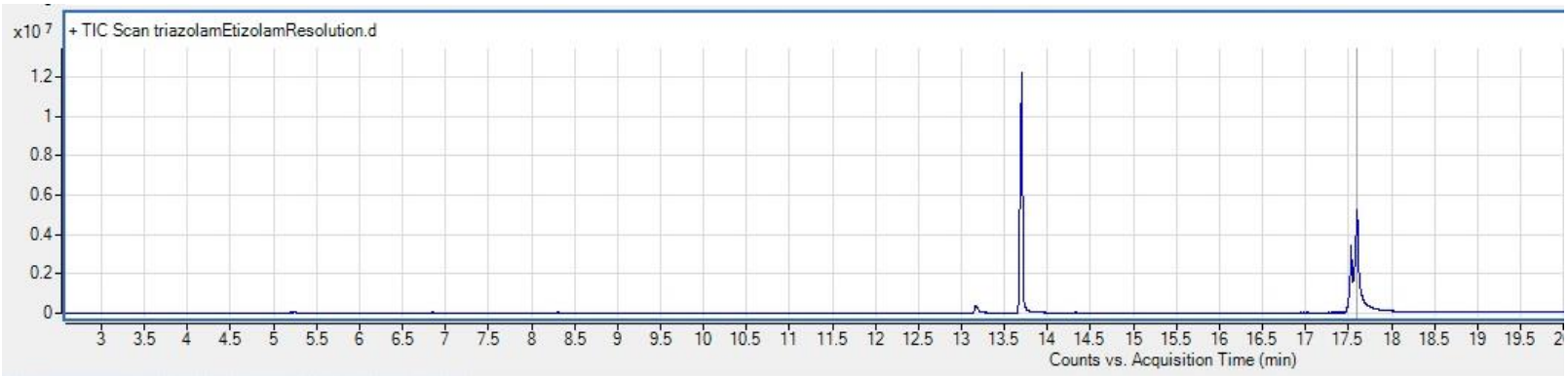


Figure 4.9. Chromatograms showing the closeness of the chromatographic peaks produced by certified standards of etizolam (7) with a Rt of 17.52 minutes and triazolam (18) at 17.59 minutes. The peaks at 13.17 and 13.72 minutes related to the internal standard, bupivacaine.

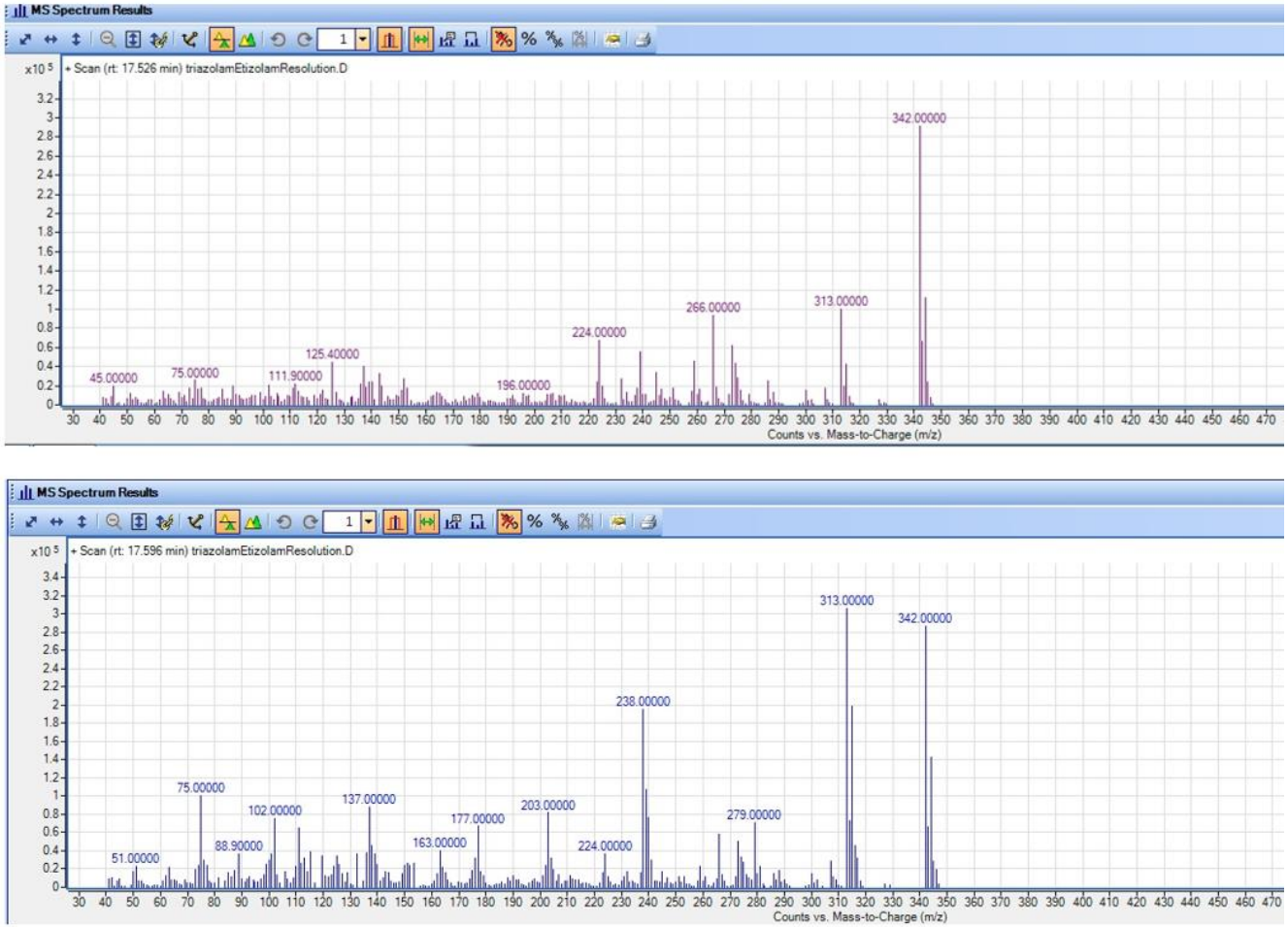


Figure 4.10. Comparison of GC-MS Rt and spectra of the etizolam (7) and triazolam (18) standards.

The upper image shows the etizolam mass spectrum and the lower shows the triazolam (18).

Using the original method described above, the pharmaceutical etizolam (**7**) standard generated a peak with the Rt of 17.52 minutes and the ions (with abundance) 342(44.0%), 313(15.0%), 266(13.2%), 224(10.6%), 137(6.2%), 75(4.4%), 102(3.5%), 45(3.1%), as listed in Table 4.2. The etizolam (**7**) standard was not recognised by the NIST 14 mass spectral library (Number 14), however consistent results were achieved using the certified standard during the analysis performed for this work. The standard was analysed at the beginning and end of each sequence containing illicit tablet samples for comparative purposes and to ensure that consistent results were obtained.

Peaks with the same Rt and ions as the etizolam (**7**) standard were identified in the illicit cases listed in Table 4.3.

Using the original method described, the certified triazolam (**18**) standard generated a peak with the Rt of 17.59 minutes, which was very close to that produced by the etizolam (**7**) standard. However, the fragmentation of triazolam (**18**) produced the ions (with abundances) 313(25.4%), 342(23.9%), 238(16.0%), 75(8.1%), 137(7.4%), 203(6.6%), 102(6.3%), 279(6.3%) which were distinct from etizolam (**7**) allowing differentiation between the two active substances. The spectra produced by the triazolam (**18**) standard was supported by the identification indicated by the NIST 14 mass spectral library (Number 14) with a suggested similarity of 93.14%.

No peaks with the same Rt and ions as the triazolam (**18**) standard were identified in the illicit cases and none are therefore listed in Table 4.2.

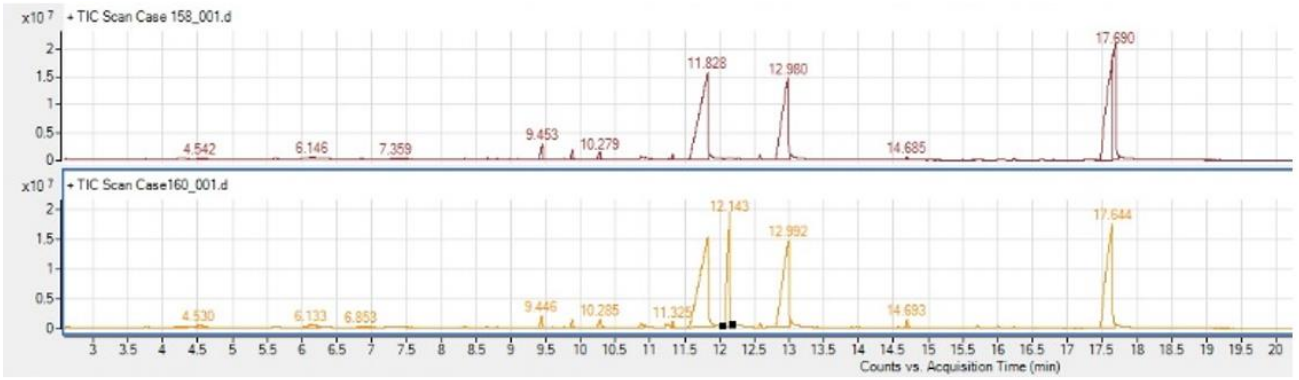
4.4.3.1 Identification of Etizolam (7)

All of the illicit cases with Rt at 17.52 – 17.55 minutes (cases 73, 76, 132, 135, 151, 155, 157, 158 and 160) were run with the mixed standard of etizolam (**7**) and triazolam (**18**). In each instance, the Rt was similar (+/- 0.03 minutes) to that of etizolam (**7**) with a corresponding match to the mass ions found in the etizolam (**7**) standard. This therefore indicated that each of these illicit cases contained etizolam (**7**) rather than triazolam (**18**).

The illicit tablets and certified standards were also analysed by HPLC to quantify the amount of active drug substance present in the illicit cases as well as build up a knowledge of the Rt of known standards using HPLC methodology. The Rt values generated by HPLC for the illicit cases was able to corroborate the original identification by GC-MS. Interestingly, the elution of the certified triazolam (**18**) standard occurred approximately 1 minute before the certified etizolam (**7**) standard and could therefore support the GC-MS distinction between the two substances (see Chapter 5 – High Performance Liquid Chromatography). A comparison of the data produced as a combination of both the HPLC and GS-MS therefore helped to give strength to the original identification of the active drug substance present in the illicit cases

In addition to etizolam (**7**), the GC-MS results indicated the presence of chlorphenamine in cases 151, 155, 157, 160, with a match in the NIST 14 library of 94% but this was not verified by further analysis. Interestingly, the remaining cases which were identified as containing etizolam (**7**) did not contain the chlorphenamine peak. The presence of chlorphenamine in the four cases, could indicate that these cases form a distinct group, with an increased likelihood of being linked, possibly by being manufactured in the same clandestine laboratory. The tablet markings of EZ/10 on the front and with a plain reverse are also only evident on these four cases and therefore supports the theory of a potential link.

The difference between these four cases and the remaining cases identified as containing etizolam (**7**) can be seen through the comparison of cases 158 and 160 (Figure 4.11). The peak at 12.1 minutes in case 160 was potentially identified as chlorphenamine with 95.27% similarity. Peaks at 11.82 and 12.98 minutes were identified as palmitic acid and stearic acid respectively. The peak at 17.6 minutes was ‘not identified’ in the NIST 14 library but matched the Rt and spectra produced by the etizolam (**7**) certified standard.



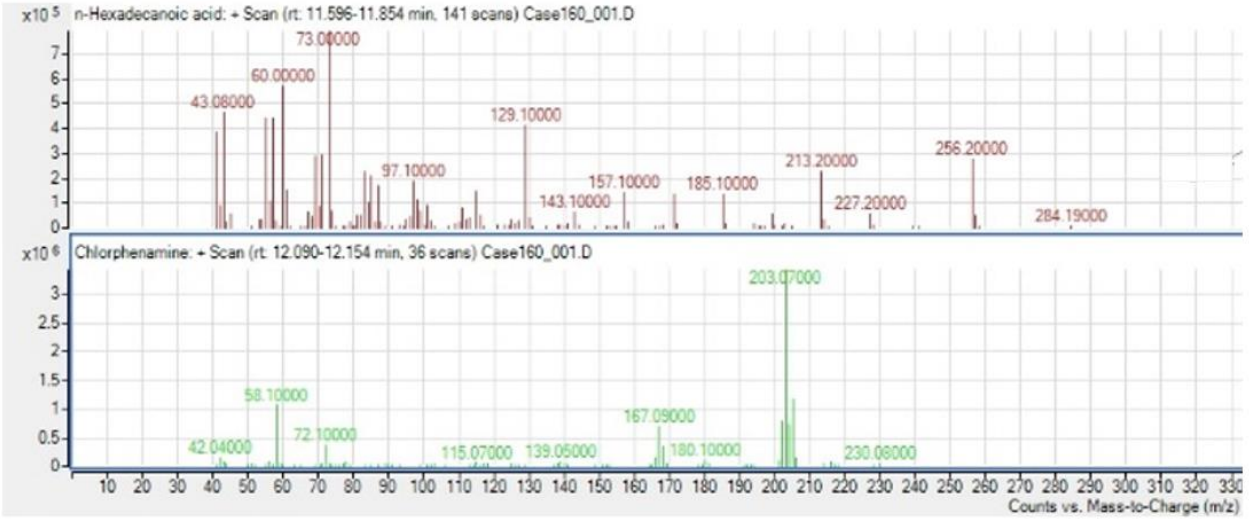


Figure 4.11. Comparison of chromatograms produced by two illicit cases containing etizolam (7) and spectra of the peaks at 11.82 and 12.98 minutes.

Although a chlorphenamine standard was not run in conjunction with case 160, to confirm the result, the spectra did correspond with that identified in the NIST 14 library (Figure 4.12).

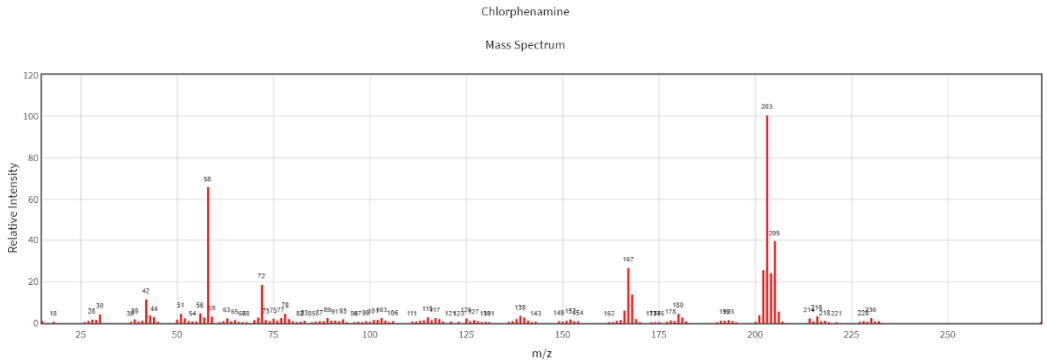


Figure 4.12. The mass spectrum of chlorphenamine shown in the NIST 14 library. (National Institute of Standards and Technology, 2017)

The physical differences in the tablet markings of EZ/1.0 on one side with a plain reverse, separated cases 151, 155, 157, 160 from the other illicit cases. The remainder of the cases containing etizolam (**7**) (cases 73, 76, 132 and 135) were marked MA and D/10 and a single case (158) marked NTZ/1.0 with a plain reverse was found to contain etizolam (**7**) as well. Interestingly, the results revealed that each of the tablet markings on all of the illicit cases, varied with the drug substance content identified through GC-MS.

GC-MS analysis also identified a small chromatographic peak in case 73, which produced the same ions (120, 92, 152, 65, 53, 81, 137, 167 in order of abundance) and had the same Rt of 6.27 minutes as the aspirin standard. This peak was not detected in the other cases containing etizolam (**7**). Although, tablets in this case had a similar appearance to those in cases 76, 132 and 135, there are several explanations for the difference. Firstly, the cases may not be related and may be manufactured completely independently of each other; secondly, inconsistent blending could result in larger quantities of constituents such as aspirin, being present in some tablets within a batch, while being of lower concentration in others even if the tablets were produced together; or thirdly, if the tablets were produced by the same manufacturer, it may be that different batches of tablets were made with varying levels of active drug substances and the cases were made at different times.

As the chromatographic peak was very small however, and given that it was only detected in the sample which was prepared using the whole tablet, it is possible that inconsistent blending leading to differences within tablets meant that the aspirin was not detected elsewhere.

4.4.4 Identification of Chlordiazepoxide (3)

In this project, the temperature of the injection port was set higher than the melting temperature of chlordiazepoxide (**3**) at 250 ^{o}C to allow vaporisation. The oven was set at 50 ^{o}C for 2 minutes before being ramped up at a rate of 15 ^{o}C / minute to 280 ^{o}C. It would therefore, have taken 17 minutes to reach the higher temperature. The chlordiazepoxide (**3**) peaks had Rt of 14.83, 15.02 and 16.67 minutes, relating to temperatures of approximately 230 ^{o}C, 245 ^{o}C and 260 ^{o}C respectively, making them lower than the degradation temperature recorded by Hida.

However, the chlordiazepoxide (**3**) certified standard produced multiple peaks when analysed by GC-MS, as indicated by the UNODC (section 4.1.2.3 'Thermal Degradation' (Division of Narcotic Drugs, 1988)) with Rt of 14.83 minutes, 15.02 minutes and 16.67 minutes (Figure 4.13).

The ions of the three peaks are listed in Table 4.2. The spectra show that the peak at 16.67 mins contains an ion consistent with the molecular ion at m/z 299. The ion at m/z 299 was not visible in the peak at 15.02 mins (Figure 4.14). It has been suggested by both Joyce (1984) and Blachut (2004), that thermal degradation is a result of the N-4 oxide in the 4-position of the 1,4-azepine ring, losing the oxygen atom in the injection port. The molecular ion of chlordiazepoxide (**3**) appears with an m/z at 298. The loss of the oxygen therefore creates an ion at m/z 282. The generation of a base peak at 282 was consistent with the analysis performed in this study and were found in the two peaks with the Rt of 15.02 and 16.67 minutes.

The mass spectrum of the chromatographic peak at 14.83 minutes shows an ion at m/z 313 which is larger than the molecular mass of chlordiazepoxide (**3**). The origin of this ion is unclear. Different preparations of the chlordiazepoxide (**3**) standard were prepared and each were found to contain this peak and blanks run between samples did not show any carry over.

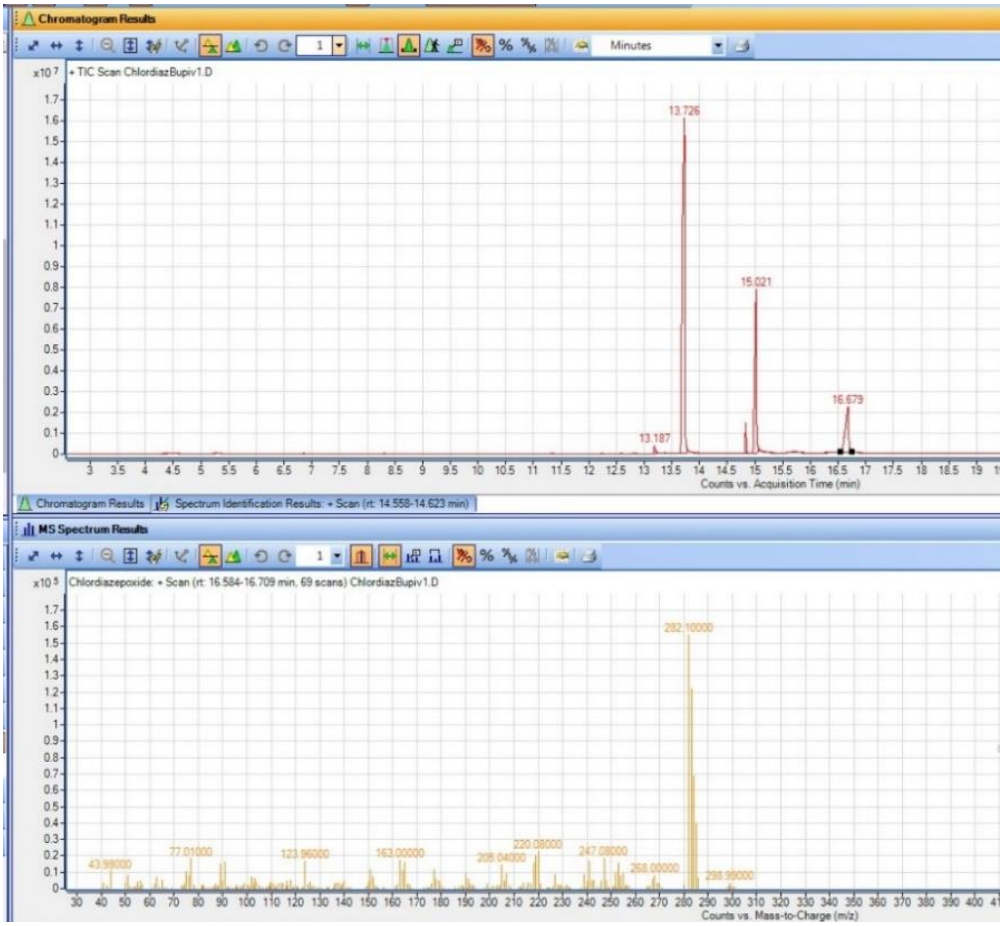


Figure 4.13. Chromatogram and mass spectrum produced by the chlordiazepoxide (3) standard. The upper image shows the peaks at 13.18 and 13.72 minutes, which relate to the bupivacaine standard isomers. The final three peaks were generated by the chlordiazepoxide (3). The lower image shows the identification of the spectrum relating to the final peak at 16.67 minutes.

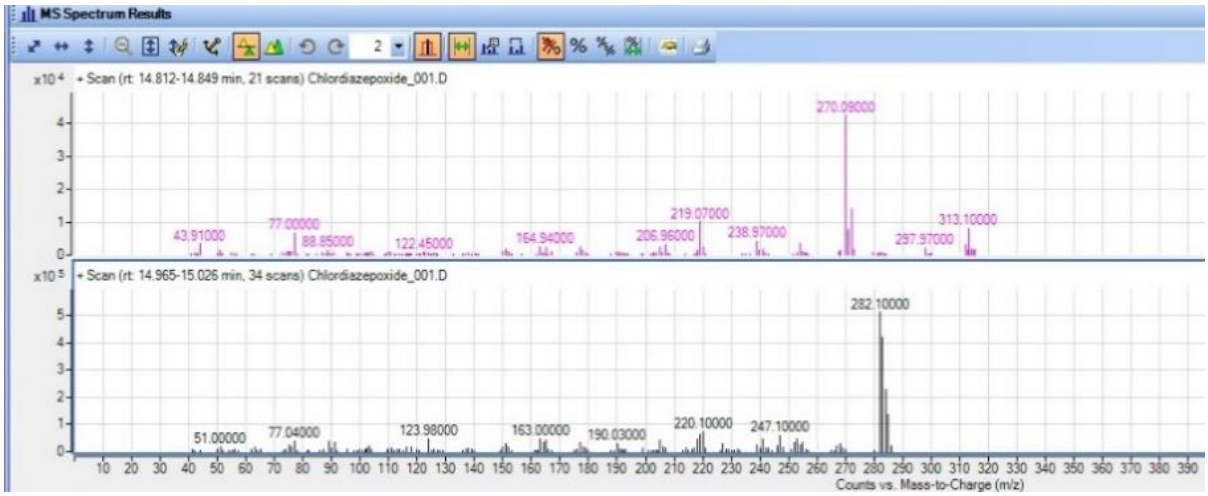


Figure 4.14. Spectra of two of the peaks produced by the chlordiazepoxide (3) standard. The upper image shows the mass spectrum of the smaller peak at 14.83 minutes and the lower image shows the spectrum of the peak at 15.02 minutes.

The origin of the chromatographic peak at 14.83 minutes with the ion at m/z 313, larger than the molecular mass of chlordiazepoxide (**3**) is uncertain and does not appear to relate to chlordiazepoxide (**3**). It could be a result of contamination, or perhaps from a rearrangement but it appeared in both the analysis of the certified standard and in samples taken from case 36. This chromatographic peak is not recorded by either Joyce or Blachut. Interestingly, the report by the United Nations Office on Drugs and Crime (UNODC) noted that chlordiazepoxide (**3**), produces multiple peaks. Although no specific details of the GC-MS results are given, the UNODC note that three peaks were detected using GC with a flame ionization detector (United Nations Office on Drugs and Crime, 2012)

4.4.4.1 Analysis of Case 136

The results produced by the gas chromatograph of Case 136, showed results consistent with a tablet containing chlordiazepoxide (**3**) (Figure 4.15), with peaks at 14.83 minutes, 15.02 minutes and 16.67 minutes, as produced by the chlordiazepoxide (**3**) standard. Interestingly, the mass spectrum for the chromatographic peak at 14.83 minutes is consistent with that from the chlordiazepoxide (**3**) standard, including the ion at an m/z of 313 (Figure 4.16). The spectra for the three peaks are shown in Figures 4.16 and 4.17.

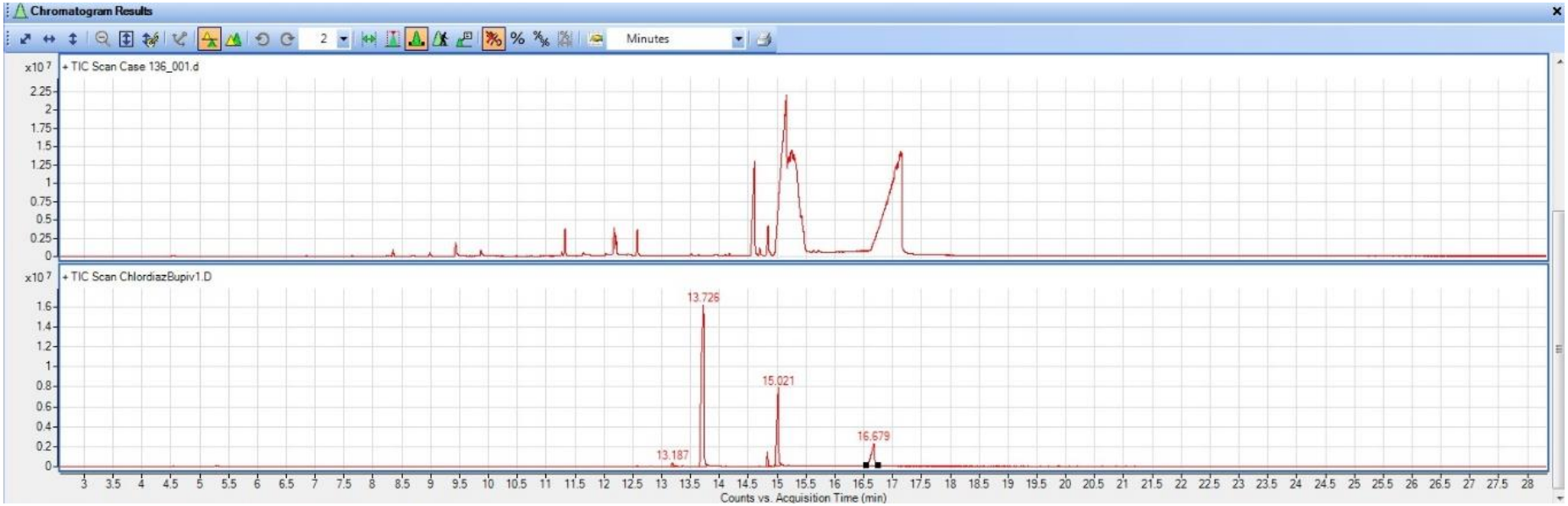


Figure 4.15. Chromatogram produced by Case 136 compared to the chlordiazepoxide (3) standard. The peaks at 13.18 and 13.72 as shown on the lower image relate to the bupivacaine internal standard.

Figure 4.16. Chromatograms and spectra of case 136. The three images at the top show the Rt of the chromatographic peaks produced by Case 136 in comparison to the chlordiazepoxide (3) standard. The two lower images show the spectra produced by the peaks at 14.82 and 15.16 minutes, which were consistent with the spectra from the chlordiazepoxide (3) standard, including the ion at m/z 313.

Figure 4.17. Two spectra produced by the broad peak at around 17 minutes generated during the analysis of Case 136.

In addition to the chlordiazepoxide (**3**), other peaks were present, which would be expected due to excipients in a tablet preparation. The smaller peaks at 9.43 and 11.32 minutes were not identified but the spectrum produced at 12.57 minutes was indicated to be methyl stearate by the NIST 14 library, with a similarity of 95.94%. Although no further work was performed to confirm this suggested identification, it would be consistent with tablet excipients.

A very small peak, barely visible above the baseline, was produced at 14.61 minutes by the chlordiazepoxide (**3**) standard, however, a much larger peak was visible on the chromatogram produced by Case 136. The spectra indicated that this was diazepam (**1**) (Figure 4.18) and was consistent with the diazepam (**1**) standard analysed by the same instrument in the same run sequence (Figure 4.19).

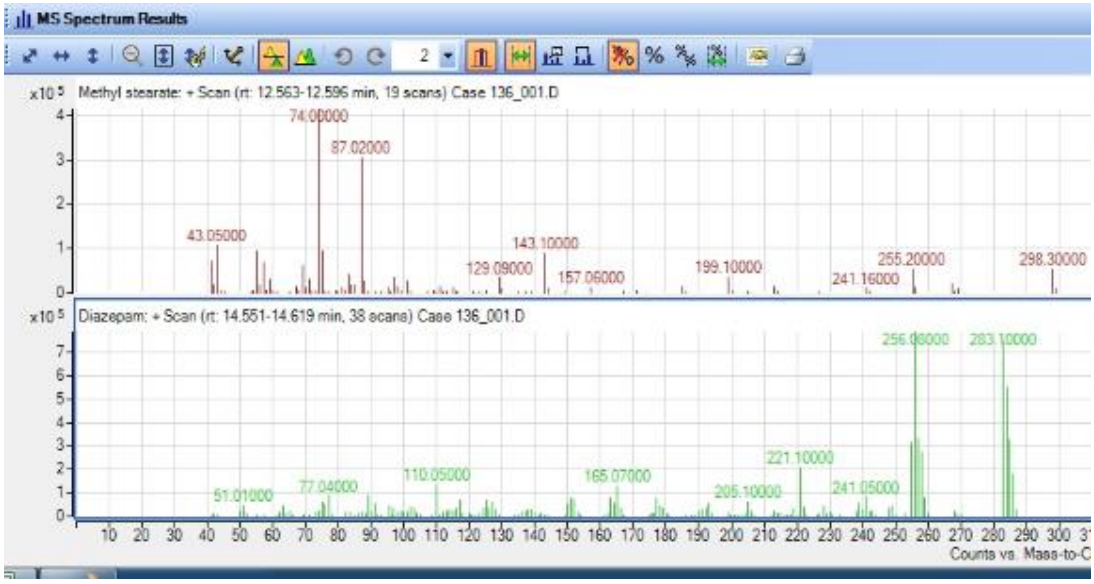


Figure 4.18. The identification of methyl stearate and diazepam (1) in Case 136.

Figure 4.19. Comparison of the chromatographic peak at 14.61 minutes in Case 136 to the diazepam (1) standard. The peaks at 13.17 and 13.72 minutes shown with the diazepam (1) standard relate to the bupivacaine internal standard.

As diazepam (**1**) is closely related to chlordiazepoxide (**3**) the chromatographic peak produced by Case 136 may be a product of degradation, as seen by the very small peak generated by the chlordiazepoxide (**3**) standard. In addition, the HPLC analysis of two different tablets taken from Case 136 indicated that the tablets did not contain diazepam (**1**). However, it may be that additional diazepam (**1**) was present and due to the illicit origin of the case, it is possible that the presence and the level of diazepam (**1**) within the tablets may be inconsistent.

4.4.5 Detection of Lubricants

Although no additional work was performed to identify excipients within the tablets, as the focus of the analysis was to identify the main active drug substance, the GC-MS analysis detected stearic and palmitic acid in 32 of the illicit cases and all of the pharmaceutical tablets sampled (Figure 4.20). These are ingredients commonly used as lubricants within the pharmaceutical industry, to reduce friction between the tablet and the tabletting machine during production (see section 2.2.4.5 lubricants, in Chapter 2 – Introduction). Although stearic and palmitic acid are not listed individually as components of the tablets on the product information leaflet for MA pharmaceutical diazepam (**1**) tablets, for example (MA Pharmachem Ltd, 2011), magnesium stearate is listed. Magnesium stearate consists of a variety of fatty acids, with over 90% content provided by a mix of stearic and palmitic acid (Delaney *et al.,* 2017).

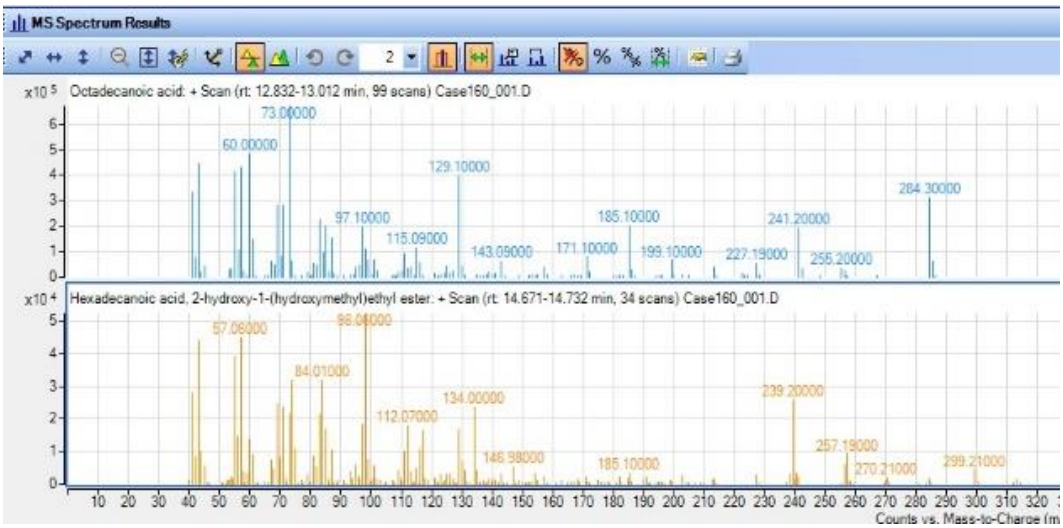


Figure 4.20. The identification of stearic acid and palmitic acid in Case 160. These are used as lubricants within the pharmaceutical industry.

Magnesium stearate is added to the pharmaceutical formula in small quantities of around 3%, due to its propensity to hinder tablet dissolution (see section 2.2.4.5 lubricants, in Chapter 2 – Introduction). Research has shown that magnesium

stearate is also present in illicit tablets, including ecstasy (Baer, 2007) and the NIST 14 library suggested similarity to chromatographic peaks detected in cases 71 and 72 (etizolam **7**) as well as 76 and 77 (phenazepam **16**), in this study, although this was not confirmed. As etizolam (**7**) and phenazepam (**16**) are not licensed for use in the UK, the identification confirms the use of lubricants in illicit tablets in this country. However, these tablets could have entered the illicit supply chain as diverted pharmaceutical preparations since they are licenced in other countries.

The use of lubricants may suggest a more advanced illicit tableting operation with clandestine producers having a knowledge of tablet manufacturing procedures. Searches of the internet reveal many websites describing how to make 'vitamin tablets and supplements' with some suggesting a formula (Ecomdash, 2019; HTC Health, 2019) and one website by London Fashion Arts sells tableting machines and provides a link to receive a free ebook on 'How to make your own tablets' (LFA Machines, 2019). London Fashion Arts also have videos on 'You Tube' to demonstrate the process (2010). The formulae described online can be adapted to contain other illicit ingredients obtained on the dark net. In addition, cheap and easily accessible pharmaceutical tablets, such as paracetamol, may be crushed and added as a bulking agent to the mix, thus providing some of the pharmaceutical constituents. However, little is known about the illicit production of diazepam (**1**) tablets and results of this analysis would indicate that cases which contain stearic or palmitic acid are not necessarily diverted, pharmaceutically manufactured products.

4.4.6 Summary of GC-MS Results

Results of the GC-MS analysis identified that 39 of the 63 seized cases analysed were found to contain diazepam (**1**). Therefore, the remaining 24 cases were presumed to be illicit. Discrimination according to main active ingredient divides the seized cases into five groups, those containing diazepam (**1**), etizolam (**7**), phenazepam (**16**), chlordiazepoxide (**3**) or promethazine (Figure 4.21). Each of the illicit cases was found to contain at least one active drug substance using the GC-MS procedure adopted in this project and on occasion additional substances such as paracetamol, aspirin, stearic and palmitic acid were also noted.

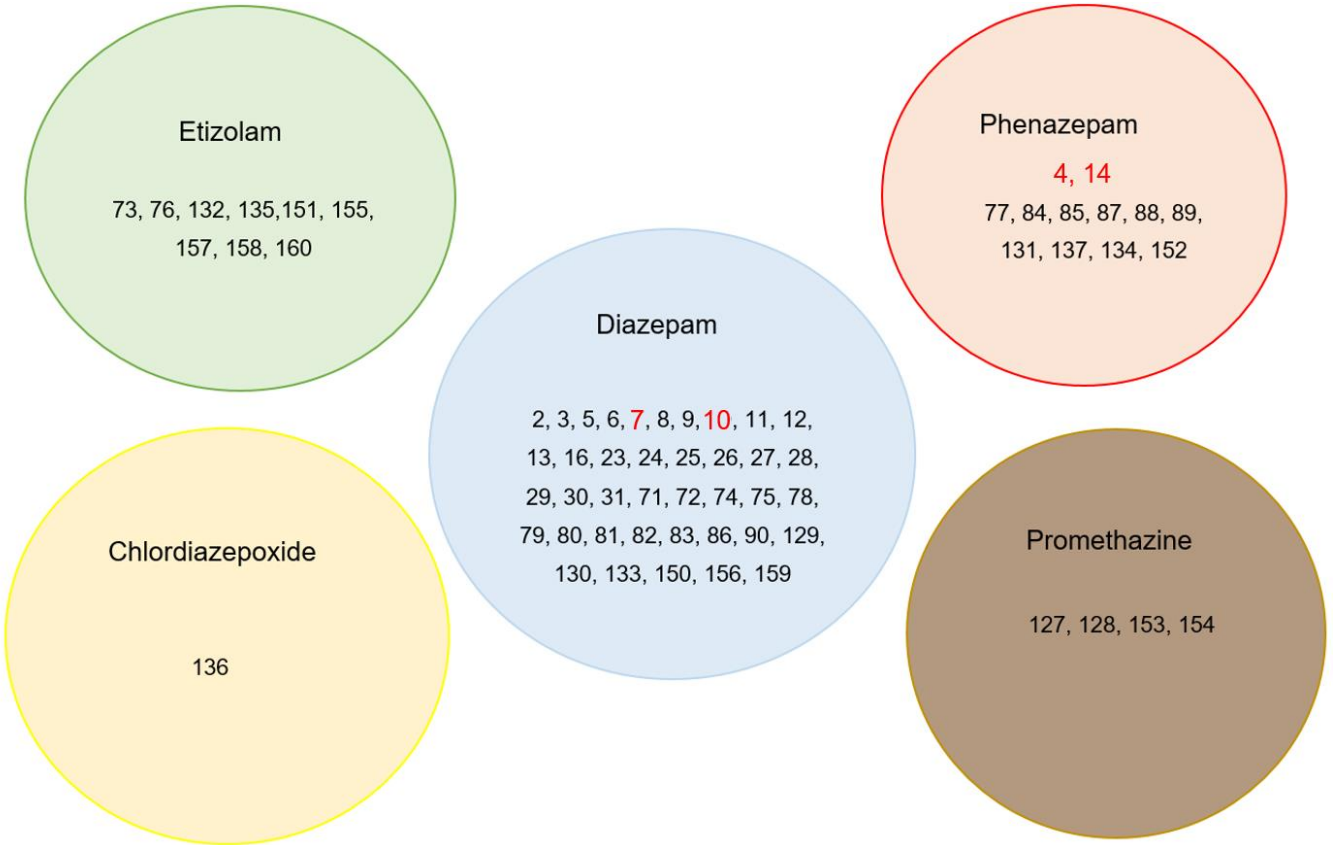


Figure 4.21. Groupings of Illicit Cases according to main Active Drug Substance.

Identification of the active drug substance present in cases 7, 10, 4 and 14 was based on pre-screening tests performed at Robert Gordon University, corroborated by HPLC and DSC results carried out as part of this project.

4.4.7 Analysis of Results

In order to assess the results of the GC-MS analysis, they were compared to the results produced from the physical examination of the tablets (Chapter 3). Knowledge gained on the main active drug substance present in the seized cases was used to give greater insight into the discrimination already performed and the results can be seen in Figure 4.22. The Venn diagram, shows the groupings indicated by the physical analysis of the tablets and further groupings which were discerned through the GC-MS. For example, the cases which had previously been identified as being similar to the pharmaceutical tablets in diameter could now be separated into groups containing diazepam (**1**), etizolam (**7**), phenazepam (**16**) and chlordiazepoxide (**3**). However, the tablets containing promethazine were shown to differ in both diameter and depth to the pharmaceutical diazepam (**1**) tablets but were of a similar weight.

Similarly, case 136, which was identified as containing chlordiazepoxide (**3**), only resembled pharmaceutical diazepam (**1**) tablets in diameter but not in depth nor weight.

Interestingly, groupings of cases containing phenazepam (**16**), which had been separated during the physical analysis, were found to show similarities to pharmaceutical diazepam (**1**) tablets in depth, or diameter, or both. However, this was shown to be distinct from tablets containing etizolam (**7**) which indicated similarities in diameter and weight but not depth.

It could be interpreted that manufacturers of these tablets could produce tablets containing phenazepam (**16**) and others with etizolam (**7**), using dies of a similar diameter, based on the results of the physical analysis. However, by examining the results of the GC-MS analysis in conjunction with physical characteristics, it could be interpreted that the manufacturer of phenazepam (**16**) tablets tend to produce tablets of similar size in diameter and depth to the pharmaceutical tablets, whereas those who manufacture the etizolam (**7**) tablets, using similar dies, are less concerned with depth but produce tablets of a similar weight. As the tablets use dies of a similar diameter, it may be that the weight is affected by differences in excipient. For

example, *Emcompress*™ (Dicalcium phosphate dihydrate), is a heavier excipient, which although present in some pharmaceutical preparations, is not used in pharmaceutical diazepam (**1**) tablets produced for the UK market. A difference in formulation between the cases, may therefore suggest that the etizolam (**7**) and phenazepam (**16**) tablets are not manufactured at the same time.

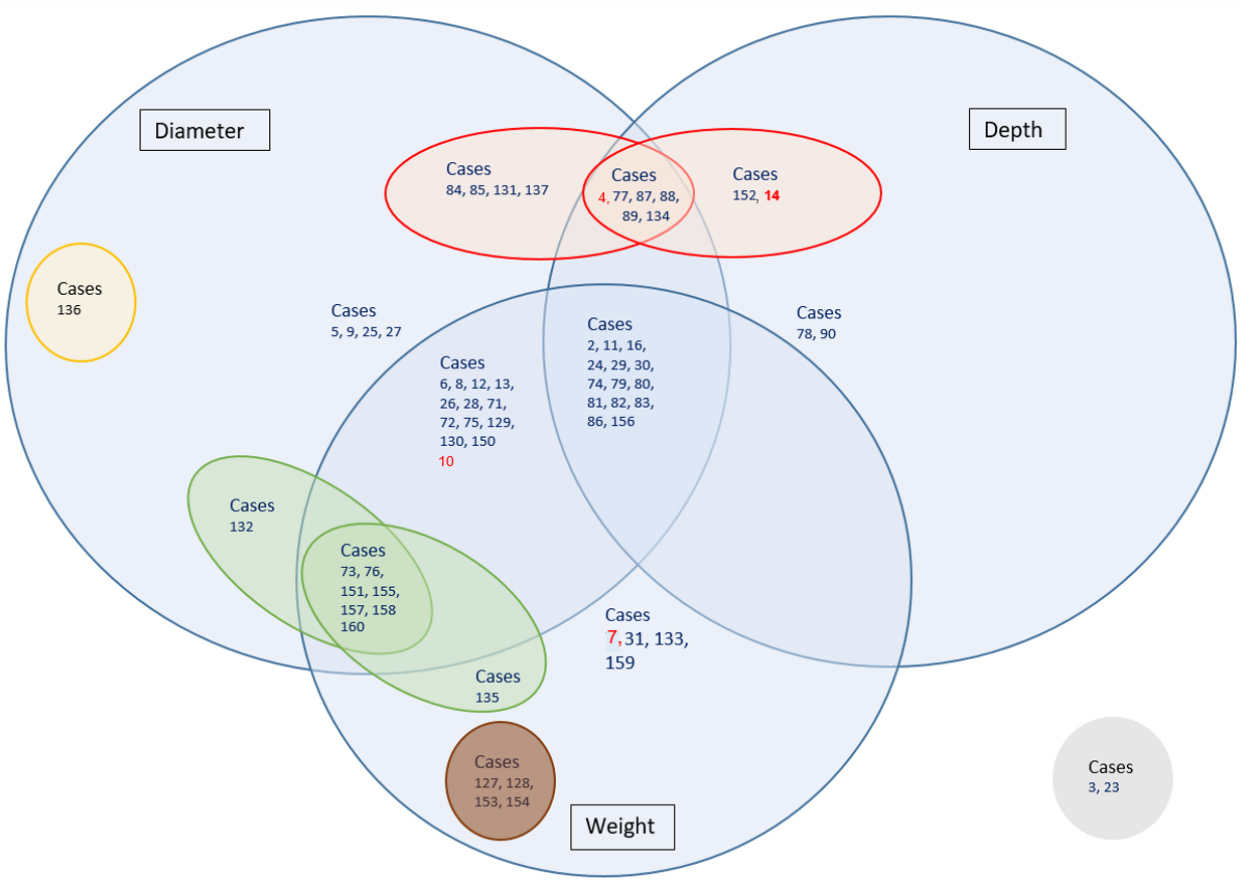


Figure 4.22. Venn Diagram of Illicit Cases according to Physical and GC-MS Analysis. Cases listed in blue contain diazepam (1). Those in red/pink shapes contain phenazepam (16), green contain etizolam (7), brown promethazine and yellow chlordiazepoxide (3). Identification of the active drug substance present in cases 7, 10, 4 and 14 was based on pre-screening tests performed at Robert Gordon University, corroborated by HPLC and DSC results carried out as part of this project.

4.5 Conclusion

For the first time, results from GC-MS analysis of real street drugs as obtained by Police Scotland have shown that there was a variety of active drug substances present in the illicit cases examined in this study. Of the 63 seized cases, which were initially presumed to contain diazepam (**1**) due to their markings and were investigated by GC-MS only 39 were actually found to contain diazepam (**1**). It is very interesting to note that the remaining 24 cases contained a variety of psycho actives many of which are illegal in the UK such as phenazepam (**16**) and etizolam (**7**). This study provides a novel snapshot of the illicit diazepam (**1**) market and provides invaluable intelligence regarding the variety of content of street drugs confiscated in Scotland. Interestingly, such seized drugs are normally not analysed to the extent as discussed in the current work and the information provided herein requires to be distributed as widely as possible as such knowledge is currently unavailable to police Scotland.

It is also worth noting that the seized cases marked MA, previously recorded as having physical measurements consistent with the known pharmaceutical tablets (Chapter 3) and subsequently found to contain approximately 10 mg of diazepam (**1**) when later analysed by HPLC, produced visually similar chromatograms to that of the known MA pharmaceutical tablets. Therefore, based on the sample preparation used in this project, it has been noted that no obvious differences exist for these particular ten seized MA cases indicating a further interesting facet of the drug chain, namely, some of the illicit drugs are probably genuine diazepam (**1**) tablets which may have been stolen, diverted or obtained by removal of quality control failures destined for destruction.

This study also presents results relating to the use and detection of lubricants in many of the illicit cases which reflects the sophistication of current illicit manufacturing methods, although some of these tablets will have been pharmaceutically manufactured tablets, which had been diverted. Initial work on the use of tablet presses and dies, has helped to link illicit cases via their mode of manufacture and circumvents the lack of quality of such produced illicit tablets which can make them difficult to recognise without extensive scientific measurement. Thus

difference in active drug substance were detected via GC-MS analysis and revealed the true nature of street drugs and gave considerable insight into the illicit market.

GC-MS analysis provided vital information for this study and the results were used to corroborate other data such as HPLC analysis and DSC. HPLC (Chapter 5) proved to be highly useful for confirming identification, for example to aid in distinguishing between diazepam (**1**) and ketazolam (**10**), where thermal degradation made absolute identification problematic. It also helped to further separate the illicit cases based on quantification of diazepam (**1**) because pharmaceutically manufactured tablets would not be expected to contain more than the "stated" dose.

DSC (Chapter 6) also provided information to support the GC-MS analysis, by generating thermal profiles with distinct differences and similarities according to the varying tablet constituents. This therefore enabled further distinction to be made between the illicit cases.

This study reports for the first time an innovative approach by utilising a variety of analytical and physical techniques to establish similarities and differences between street drugs. The use of GC-MS was invaluable at identifying more unusual active ingredients and could lead to methodology which could be expanded to provide drug intelligence for Police Forces worldwide.

Chapter 5. High Performance Liquid Chromatography

5.1 Chapter Summary

High Performance Liquid Chromatography (HPLC) was used to quantify the diazepam (**1**) content of three tablets taken from each seized case and from each of the pharmaceutical batches, used for comparative purposes. Although the seized cases contained tablets which were blue in colour, consistent with the colour of 10 mg diazepam (**1**) tablets manufactured for the UK market, some of the cases were identified as containing other active drug substances through GC-MS analysis (Chapter 4). HPLC analysis was therefore used to further distinguish between tablets by identifying the seized cases which contained a level of drug content consistent with 10 mg diazepam (**1**) tablets manufactured for the UK market. However, the presence of the correct amount of active drug substance does not necessarily mean the tablets have been pharmaceutically manufactured.

5.2 Introduction

5.2.1 Uses of HPLC

5.2.1.1 HPLC in Pharmaceutical Studies

High Performance Liquid Chromatography (HPLC) is an important method of drugs analysis. It is used in pre-formulation studies within the pharmaceutical industry, as well as in the testing of illicit drugs. However, until now, no work has been performed by HPLC, to quantify the amount of active drug substance present in illicit blue tablets with markings consistent with pharmaceutical diazepam (**1**) tablets. This was partly because of the large numbers of pharmaceutical tablets that were being diverted into the illegal supply chain, as described by the Advisory Council on the Misuse of Drugs (2016), leading to an assumption that many of the diazepam (**1**) products in circulation were diverted products of pharmaceutical origin (United Nations Office on Drugs and Crime, 2012).

In pre-formulation studies HPLC is utilised for the identification and quantification of impurities and degradation products (Narang, Desai and Badawy, 2012).

Wyttenbach, at Hoffmann La Roche, used the level of impurity found through HPLC analysis, as a means of determining the amount of drug degradation that had taken place (Wyttenbach *et al.,* 2005). Information on degradation products and impurities can also be of interest forensically and could potentially help to link different cases. However, matching retention times alone, does not necessarily mean that the same unknown substances are present. Whereas, the benefit of using this technique for pharmaceutical testing is more palpable since a specific set of known impurities is often studied to provide assurance that there has been no significant impact on the efficacy of the material.

An interesting study in Nigeria compared a variety of pharmaceutical ibuprofen tablets to investigate their equivalence. This included ensuring that each of the manufacturers produced tablets that conformed to the regulations stipulated in the British Pharmacopoeia, regarding everything from tablet size, disintegration and dissolution rates to the level of active drug content. The results of the study revealed that although the level of active drug substance may be similar, differences in physicochemical characteristics such as dissolution rates, could greatly affect the bioavailability of the ibuprofen. The differences were likely to originate from variation between excipients. Quantification of the active ingredient for the study, was performed on HPLC in addition to UV assay. The results indicated that despite all of the tablets falling within the guidelines, there was some variation between the pharmaceutically manufactured tablets. However, it was noted that although the results generated by the HPLC demonstrated that all of the tablets did satisfy the regulations, the results produced by the UV assay did not and suggested that two of the brands contained a lower level of ibuprofen, at 92.43% and 91.18% compared to the HPLC indications of 100.54% and 96.66% respectively. The results of the UV assay were not consistently lower with 50% indicating a higher concentration of ibuprofen than by HPLC analysis. It was suggested that this was because the HPLC was a more consistent and sensitive technique (Eraga *et al.,* 2015).

5.2.1.2 Analysis of Illicit Tablets

In terms of illicit drug analysis, HPLC can help to corroborate identification of the active drug substance by comparing the retention time to a known standard, once

the substance has been identified by gas chromatography – mass spectrometry (GC-MS), as well as providing a reliable method of quantification. This therefore makes it an invaluable technique for the analysis of illicit tablets.

A study in 2011 compared the use of reversed-phase HPLC with capillary zone electrophoresis (CZE) for the separation of benzodiazepines. In this experiment, a Zorbax Stable Bond C-18 column was compared to a C-8 column using a mobile phase of acetonitrile and deionised water with acetic acid, at a pH of 3.0. It was discovered that using the C-18 column reduced the retention time as a higher proportion of acetonitrile could be incorporated into the mobile phase. In comparison to the CZE, HPLC was the preferred technique because the level of baseline noise was lower. In addition, the increased noise in combination with the smaller sample size for the CZE resulted in a much higher Limit of Detection (LOD) and Limit of Quantification (LOQ) (Kalíková *et al.,* 2011).

Although, Kalíková described how retention times could be reduced by increasing the amount of acetonitrile in the mobile phase, the shorter chain length in C-8 columns also gives a lower retention time (Agilent Technologies, 2005). For this research a C-8 column was used as good separation was achieved with the lower retention time.

5.2.2 Methodology

5.2.2.1 Methodological Background

It was noted by the United Nations Office on Drugs and Crime (UNODC) that a specific methodology for use with the HPLC would not be appropriate for the analysis of all benzodiazepines. This was due to the range of chemical structures within the drug family. However, it was suggested that the drug sample being tested should be prepared in methanol, which could dissolve the drug substance whether in free base or salt form. The recommended method included the use of a mobile phase consisting of methanol, water and phosphate buffer. The proposed injection size was 20 µl, using a UV detector set at 240 nm. (United Nations Office on Drugs and Crime, 2012).

The method described by the UNODC was designed for the general analysis of benzodiazepines. As the aim of this project was to analyse illicit blue tablets believed to be sold as diazepam (**1**), the adopted HPLC method used was based on the European Pharmacopeia Monograph for the purity assessment of diazepam (**1**) drug substance. To ensure that the recovery of drug substance was complete the preparation of tablets for UV assay (B.P. monograph) using acidified methanol to quantitatively extract the diazepam (**1**) from the formulation was followed, with a mobile phase of 22: 34: 44 acetonitrile: methanol: potassium dihydrogen phosphate solution.

The eluent was run as an isocratic system with a flow rate of 1 mL / minute and a run time of 15 minutes.

The report by UNODC also suggested that multiple tablets should be combined and analysed together for quantification purposes. However, this was based on the assumption that the illicit tablets had been pharmaceutically manufactured and diverted into the illegal supply chain. Quantification of multiple tablets was not believed to be appropriate for this project, because the differences in active drug substance as well as the varying levels present, indicated that the tablets had been illicitly manufactured and may not be uniform. This therefore meant that different tablets within a case could potentially contain disparate active ingredients or be inconsistent according to the effectiveness of the blend (United Nations Office on Drugs and Crime, 2012).

After reviewing a variety of detectors, Siddiqui commented that UV detection is often preferred as it provides the best specificity, reliability and repeatability with a short analysis time (Siddiqui, Alothman and Rahman, 2017). However, process or degradation impurities may have a similar structure and absorb at the same wavelength as the drug substance (Ryan, 1998), therefore producing a bigger peak by UV.

Single wavelength analysis can provide excellent results when the corresponding wavelength is known and the European pharmacopeia monograph advised using a UV detector set to a wavelength of 254nm. The use of a diode array detector would

have given confidence in the peak purity of the HPLC diazepam (**1**) peak, indicating that the chromatographic peak was only due to diazepam (**1**) and no other substance. However, as a UV detector was used for this study, GC-MS was used to confirm the drug substance and determine the likelihood of co-elution on the HPLC. In addition, comparison of retention times with the appropriate drug standard was constantly performed by HPLC analysis.

5.2.2.2 Sample Sizes

In the 'Guidelines on Representative Drug Sampling' produced by the United Nations Office on Drugs and Crime, several methods for determining a suitable number of samples were suggested, including batch analysis according to markings. For police seizures, it indicated that each situation should be case dependent (2009). Izenman wrote a detailed analysis of representative sampling in America and noted how an Illinois court supported the decision of a chemist to analyse three tablets in a batch of 100 for identification purposes (Izenman, 2003). The tablets received for analysis in this study were themselves just a representative amount of each police production sampled. Entire cases of small productions were obtained but when larger cases were seized by the police, a selection of the visually similar tablets were provided for the project.

The tablets analysed for this study were divided into separate seized cases. Many of the seizures were small, with 29 of the seized cases containing fewer than ten tablets for analysis and the remaining 36 seized cases contained between 20 and 70 tablets. The hypergeometric distribution method, uses a frequentist approach to determine the number of variations in drug substance within a batch, depending on the number of tablets analysed and the number of samples containing a variation. In order for a 95% confidence interval (making a correct assumption for about 95% of the entire batch), a sample size of three would need to be analysed in cases containing fewer than ten tablets. For cases containing between 10-70 tablets, four tablets were suggested (United Nations Office on Drugs and Crime., 2009), For this project, three illicit tablets taken from each case were analysed by HPLC to ensure the peak produced by the active drug substance present in the illicit tablets matched the retention time of the diazepam (**1**) standard. The scientific assumption was made

that all tablets within a case would be similar in terms of quantification, unless analysis proved otherwise. This allowed an average result to be reported for each case, given that in most cases the results were very similar. Reproducibility was determined by preparing three tablets from each case, with three vials made from each tablet solution and three readings taken from each vial, thus giving confidence in the individual results. This was further supported by comparison of the GC-MS and DSC analysis.

5.3 Experimental

5.3.1 Instrumentation

The HPLC analysis was performed on a Shimadzu 10 HPLC system with autosampler and ultra-violet detector set at 254nm. This was supported by Clarity software.

The column used was a Zorbax XDB C8 Eclipse column of 4.6 x 150mm, with a 5µm particle size and double end-capped, purchased from Agilent Technologies. This was maintained at 30 ^{o}C in the column oven.

5.3.2 Materials used in the HPLC Analysis

Hplc grade acetonitrile, ethyl acetate, hplc grade methanol and potassium dihydrogen phosphate were obtained from Fisher Scientific, Loughborough. Chlordiazepoxide (**3**) certified standard 1 mg / mL in methanol, diazepam (**1**) certified standard 1 mg / mL in methanol, etizolam (**7**) certified standard 1 mg / mL in methanol, triazolam (**18**) certified standard 1 mg / mL in methanol, acetaminophen 1 g analytical standard, aspirin 1 g analytical standard and bupivacaine 1 g analytical standard were purchased from Sigma Aldrich, Gillingham. Ketazolam (**10**) certified standard 1 mg / mL in methanol and phenazepam (**16**) certified standard 1 mg / mL in methanol were provided by LGC, Middlesex. MA tablets (supplied by MA Pharmachem Ltd., Bolton, UK), Wockhardt tablets (supplied by Wockhardt UK Ltd., Wrexham), Teva tablets (supplied by Teva UK Ltd., Runcorn), Actavis tablets

(supplied by Actavis UK Ltd., Barnstaple) and illicit blue tablets (supplied by Police Scotland).

5.3.3 Preparation of Solutions

The method used acidified methanol to quantitatively extract the diazepam (**1**) from the formulation, with a mobile phase of 22: 34: 44 acetonitrile: methanol: potassium dihydrogen phosphate solution.

500 mL of a 3.4 g / L solution of potassium dihydrogen phosphate was prepared and adjusted to pH5 with a 0.1 M sodium hydroxide. The resulting solution was then 5 times the strength of the buffer required for the final mobile phase but since the storage was better than for a more dilute buffer, this was then diluted appropriately with distilled water immediately before adding to the mobile phase.

The mobile phase was prepared by adding the prepared diluted potassium dihydrogen phosphate to a conical flask, containing methanol and acetonitrile and the solution was de-gassed with a vacuum filter, before use.

This eluent was used as the mobile phase, for the dilution of samples and for the blanks.

5.3.4 Preparation of Standards

Each standard was prepared separately. 1 mL of the certified 1 mg / mL diazepam (**1**) drug standard was pipetted into a 10 mL volumetric flask and made up to the mark with methanol. This was the working 0.1 mg / mL stock solution. The standards were prepared for the calibration of 0.0025 mg / mL, 0.01 mg / mL, 0.02 mg / mL, 0.03 mg / mL and 0.05 mg / mL dilutions.

In addition, a further 0.02 mg / mL diazepam (**1**) standard was prepared and used at the beginning and end of each run sequence in order to monitor drift and were run before and after corresponding samples from the illicit cases which had been identified by Gas Chromatography - Mass Spectrometry. A 0.02 mg / mL solution was also prepared from each of the certified standards of other active drug substances identified through GC-MS analysis to run at the beginning and end of

each run sequence containing the corresponding drug substances as a comparison for the seized tablets and to identify the R_t of the known standards on the HPLC.

5.3.5 Preparation of Tablets

Both pharmaceutical and illicit tablets were prepared in the same way. This involved softening the tablet in 5 mL of distilled water and then dissolving the drug content in approximately 40 mL of 0.5% w/v solution of sulphuric acid in methanol.

The solution was agitated with a flask shaker for 15 minutes before being made up to 100 mL with additional acidified methanol. The agitate solution was then filtered into a flask to remove any insoluble material and an aliquot (2 mL) of the filtered solution was pipetted into a 10 ml volumetric flask and made up to the mark with eluent, giving a further 1 in 5 dilution. This was passed through a syringe fitted with 4mm filter (0.2µm pore size) into an HPLC vial. The sample was introduced into the HPLC as a 20 µL injection.

Some of the tablets from the illicit cases were still outwith the linear working range of the method and required a further 1 in 25 dilution, which was analysed with a 20 µL injection, resulting in a multiplication factor of 2500.

5.3.6 Validation of the Method

The linearity of the method was determined by making up five solutions with different concentrations across the range 0.0025 mg / mL to 0.05 mg / mL and provided a validation for up to 25 mg of diazepam (**1**). Each of the solution concentrations were prepared three times to determine the method precision. The area response was plotted against concentration and least-square regression was used to determine slope, intercept and correlation coefficient. The resulting calibration curve (Figure 5.1) had a correlation coefficient of 0.9994 with an equation of the line determined by the calibration curve. The equation of the line was used to calculate the concentration of diazepam (**1**) mg /mL, within each sample. So that:

$$x = \frac{mAUs + 71.836}{301833}$$

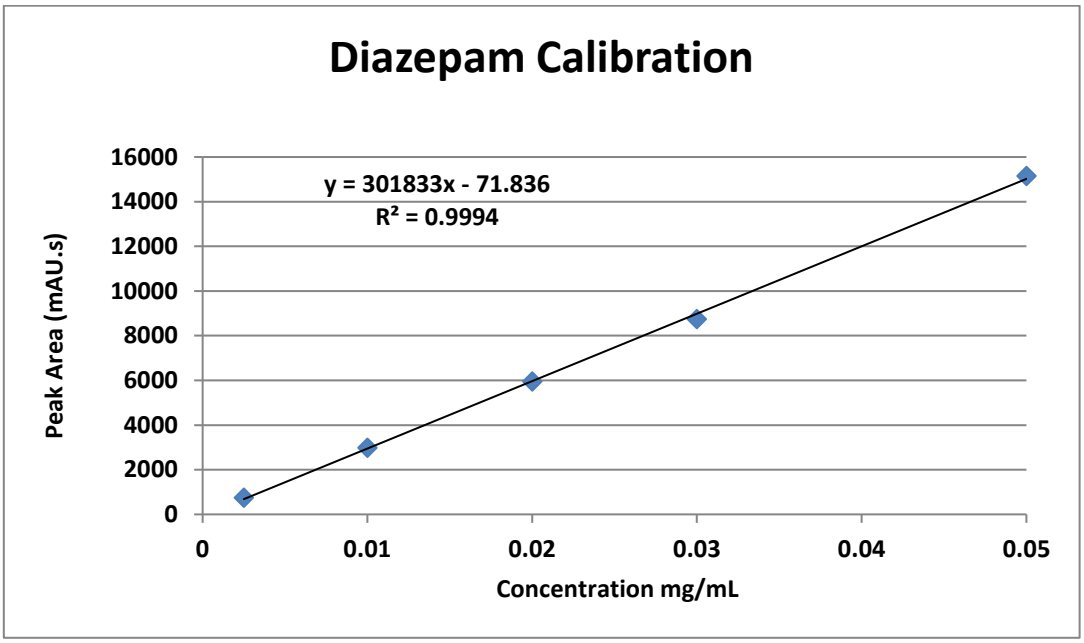


Figure 5.1. HPLC calibration curve.

Precision was determined on six runs of the 0.02 mg / mL standard using a 20 µL injection and the coefficient of variation at 0.02 mg / mL was found to be 0.31%, as shown in the Table 5.1:

Table 5.1. Results of the coefficient of variation, based on six runs of a 0.02 mg / mL standard.

Injection Number	mAUs Detected
1	5981.532
2	5983.214
3	5968.134
4	5932.729
5	5961.267
6	5955.535
Mean	5963.74 mAUs
Standard Deviation	18.71 mAUs
Coefficient of variation = Standard Deviation / Mean x 100	= 0.31%

The recovery of the method was assessed by spiking 2 mL taken from a 0.05 mg / mL sample prepared from a known 5 mg pharmaceutical tablet with 0.5 mL of a 0.1 mg / mL standard solution and making up to the mark in a 10 mL volumetric flask, to give a final concentration of 0.015 mg / mL. The milli-absorbance units (mAUs) obtained were put into the equation of the line determined by the calibration curve:

$$x = \frac{mAUs + 71.836}{301833}$$

A mean recovery of 0.0141 mg / mL was obtained from three 0.0150 mg / mL samples analysed. This calculated to a final recovery of 94%:

Injection 1: mAUs recovered = 4197.583

Therefore: $x = \frac{4197.583+71.836}{301833} = 0.0141449$

Injection 2: mAUs recovered = 4196.315

Therefore: $x = \frac{4196.315+ 71.836}{301833} = 0.0141408$

Injection 3: mAUs recovered = 4198.145

Therefore: $x = \frac{4198.145+71.836}{301833} = 0.0141468$

Mean = 0.0141442

Amount of diazepam (**1**) in sample = 0.015 mg / mL

Therefore 0.0141442 / 0.015 = 0.9429

x 100 = 94%

Sample solution stability was studied at 24, 48 and 120 (due to a weekend) hour intervals, using samples both stored in the autosampler at room temperature and in the refrigerator set at 4 ^{o}C, with consistent results being achieved. Finally, the accuracy of the process was demonstrated by analysis of known pharmaceutical grade tablets, which allowed the calculated experimental values to be compared with the anticipated amounts.

Limit of detection (LOD) was determined by analysing a series of dilutions down to 0.000005 mg / mL. The smallest level detected was found to be 13 ng / mL. This gave an LOD calculated using the equation (Swartz, 2006):

$$\frac{3.3 \text{ x standard deviation}}{\text{gradient of the line}}$$

The results of the three samples detected were:

Sample Number	mAUs Detected
1	7.278
2	9.450
3	7.424
Mean	8.050667mAUs
Standard Deviation	1.214055mAUs

therefore:

$$\frac{3.3 \text{ x } 1.214055}{301833}$$

$$= 0.00001327 \text{ mg / mL}$$

$$= 13 \text{ ng / mL}$$

and a limit of quantification (LOQ) calculated using the equation (Swartz, 2006):

$$\frac{10 \text{ x standard deviation}}{\text{gradient of the line}}$$

$$\frac{10 \text{ x } 1.214055}{301833}$$

= 0.00004022 mg / mL

= 40 ng / mL

5.3.7 Precision of Quantification

Due to the larger numbers involved, it was calculated that the highest standard deviation from three replicates was produced by the 0.05 mg / mL standard, with a standard deviation of 76.14 milli-absorbance units (mAUs). The 95% confidence interval level of two standard deviations was used for acceptance of the results and was calculated at 152.28 mAUs. This value was put into the equation of the line as the y value and multiplied by the appropriate dilution factors, in order to determine what the +/- allowance values would be in terms of mg / mL. After taking into account the dilutions used in the tablet preparation, this equated to an allowance of ±0.37 mg per tablet.

Given that the tablet preparations had an initial dissolution factor of 100 followed by a one in five dilution to bring the sample solution within the calibration range for a 20 µl injection, then this also meant that any errors would be subject to a multiplication factor of 500. Clearly, this does have an impact on the reported quantification results and all results were initially rounded to one decimal place but reported to the nearest whole number.

In an attempt to monitor the variation of results for any one tablet, three vials were prepared from each tablet and three injections run from each vial. In order to understand within case variation, three tablets were prepared from each case following the above process. However, an awareness of the possibility of error must be considered when accepting the results of quantification.

Although potential errors and large multiplication factors may impact on the quantification of diazepam (**1**), it is believed that an adequate assessment of drug content was achieved for the purposes of this project. The results ultimately showed a variation in diazepam (**1**) levels between around 8 – 48 mg in tablets that were anticipated as containing 10 mg. Any greater precision and accuracy would have

required far greater input of time and use of standards and been largely unnecessary for what was being attempted in this project. Differences in concentration of around 0.5 mg would have added little further information given that the pharmaceutical diazepam (**1**) drug release specification states that the level of active drug substance should be 10 mg +/- 7.5%, equating to 9.25 mg to 10.75 mg (British Pharmacopoeia Commission, 2017b). In addition, the quantification of diazepam (**1**) was only one of the parameters being studied for this project and the aim was to combine scientific results to provide a greater understanding of the illicit seized tablets.

5.3.8 Run sequence

Samples were run with blanks of eluent between vials, in order to avoid carry over between samples and to detect long retention times. Standards were run at the beginning and end of each sequence to monitor drift and ensure daily accuracy. In addition to the suitability check at the start and end of the run additional checks were made between tablets of the same case. In total three tablets from each case were normally analysed by HPLC. Three vials were prepared of each tablet solution and three different analyses were made from each vial, giving nine results for each tablet analysed and 27 results for each case.

5.3.9 Overview of the HPLC Analysis

5.3.9.1 Drift in Retention Times

The retention times were found to drift on occasion. This appeared to be caused by the age of the mobile phase, the fluctuating temperatures in the laboratory and by moving the location of the instrumentation. The mobile phase was therefore changed weekly and was kept as consistent as possible for comparative purposes. In addition, a diazepam (**1**) drug standard was run at the beginning and end of each sequence to monitor any small drift that occurred each day.

5.3.9.2 Difference in Colourants

As each tablet was softened in water and acidified methanol, it was noted that there were a variety of differences in the solutions produced. This was not only true of the illicit cases but also the different manufacturer's pharmaceutical preparations. For

example, the Actavis and Teva tablets produced a clear solution with insoluble blue particulates in the bottom of the flask, whereas the MA tablets produced a pale blue solution with a few fine residual white particulates. This would suggest a difference in the colourant used. MA Pharmachem use the water soluble indigo carmine dye (E132) in their manufacturing process (MA Pharmachem Ltd, 2011). Whereas, Actavis 10 mg diazepam (**1**) tablets contain the HT lake (E132) version of the same colour. The aluminium lake is a more stable dye, which is not water soluble and is often used in the food industry (Actavis UK Ltd, 2014). Lakes are formed by the precipitation of a food dye with an aluminium salt, making them virtually insoluble in water and they therefore colour by dispersion (Pharmorgana GmbH, 2018). Although the Teva tablets dissolved to produce a clear solution with insoluble blue particulates, very similar to Actavis, the patient information leaflet only identifies the dye E132 without detailing whether this is in lake form (Medicines and Healthcare Products Regulatory Agency, 2015b).

Results indicated there was more fluctuation in the level of diazepam (**1**) content found within the Actavis and Teva tablets during the initial analysis in this study. The problem may have been due to incomplete dissolution of the diazepam (**1**) with some active drug substance becoming trapped in the larger particulate material which was removed during filtration. Replacing the filtration step by centrifuging the samples at 3000 RPM for 10 minutes, a more consistent level of diazepam (**1**) at around 10 mg was then recovered from the pharmaceutical tablets. As a result, the centrifuge was then used on all cases, in order to alleviate any discrepancies.

5.3.9.3 Tablet Availability

Given the small number of tablets within many of the batches, three tablets were normally used for HPLC analysis. However, due to limited numbers of tablets, only one tablet was analysed by HPLC from Cases 2 – 31 as the few remaining tablets were required for other forms of analysis. Limited availability of later cases meant the cases that were found not to contain diazepam (**1**) in the initial HPLC analysis were identified by GC-MS and re-run on the HPLC along with a standard of the indicated drug substance.

Although certified drug standards were run before and after each run of pharmaceutical and illicit tablets to ensure retention times were correct for the suspected substance, on occasion, this was not possible. For example, the limited number of tablets available for Cases 4 and 14 meant that the initial HPLC was performed to provide an indication of retention time. However, once the certified standards had been obtained there were no tablets left to compare them to. The active drug substance was then tentatively identified by matching retention times with other cases run at the same time and then later matching them with the certified standards. The recorded retention time for the visible peaks produced by the earlier run of Cases 4 and 14, were also compared to the later standards. DSC thermograms were also compared to gain additional information. This therefore may give an indication of the active drug substance present but is not conclusive.

5.3.9.4 Consistency of Blend of the Illicit Tablets

In order to investigate the uniformity of the active drug substance within one of the illicit tablets, a single tablet from Case 27 was quartered and analysed. A content uniformity test was performed by Zaid et al. (2013) on ½ scored pharmaceutical lorazepam (**11**) tablets. Lorazepam (**11**) tablets were chosen because of the popularity of benzodiazepines and as there was a small amount of active ingredient within the tablet. Essentially this would mean that if the tablets were inconsistent in the distribution of active drug substance when half tablets were the prescribed dose, which Zaid suggested was common practice, then incorrect amounts may be taken. The results indicated that the analysed pharmaceutical tablets were evenly blended with consistent levels of lorazepam (**11**) in each tablet half.

The situation was very different for this project. Case 27 had the highest recorded level of diazepam (**1**) found in this study, with 48 mg reported in a single tablet. The test was performed to investigate whether each quarter of the tablet contained an equal amount of the diazepam (**1**) content.

However, as this was an illicitly manufactured tablet, it may be that if this tablet had a high diazepam (**1**) content then others in the batch may have been low, depending on how well mixed the ingredients had been prior to tableting. It is worth pointing out

however, that several of the tablets from this case had been analysed both during this study and for honours projects and the quantity of diazepam (**1**) present had been consistently high. Also, the results of this case may not necessarily reflect on the effect of batch mixing for other illicit cases.

Although recreational drug use often involves the consumption of large quantities of multiple drugs, the option of internet purchases for medicinal use in countries such as America, may mean that half tablets are consumed. It would therefore be possible that a person splitting what is believed to be a 10 mg diazepam (**1**) tablet to provide a 5 mg dose, could be taking 20 mg of an unequally blended illicit tablet, for example.

5.4 Results and Discussion

5.4.1 Content of Illicit Cases

5.4.1.1 Active Drug Substances Detected

The results of the HPLC analysis are shown in Table 5.2. Results were rounded to give a reported level for statistical analysis. However, cases found to contain between 9 – 11 mg were all reported as containing the 10 mg pharmaceutical dose to allow for errors of measurement and the 7.5% tolerance permitted within the pharmaceutical industry.

The results indicate that of the 65 cases of illicit tablets analysed 31% (20 cases) were found to contain the 10 mg stated pharmaceutical dose of diazepam (**1**) (approximately 9 – 11 mg); 3% (2 cases) contained a slightly lower amount (approximately 8 mg); 26% (17 cases) contained considerably higher than the expected dose (19 – 48 mg); and 40% (26 cases) were found to contain no diazepam (**1**) but other active drug substances instead.

Table 5.2. Results of the HPLC analysis. The tablet marked * was used to check the homogeneity of a quartered tablet.

	Diazepam (1) content recorded in tablet (mg)			Reported Level	Mean	Standard Deviation	Relative Standard Deviation
Case Number	Tablet 1	Tablet 2	Tablet 3				
2	9.5	-	-	10	9.5		
3	8.9	-	-	9	8.9		
4	0	-	-	0	0		
5	7.8	-	-	8	7.8		
6	27.7	-	-	28	27.7		
7	22.3	21.0	-	22	21.65	0.9	4.3
8	20.4	-	-	20	20.4		
9	21.9	-	-	22	21.9		
10	9.9	-	-	10	9.9		
11	9.6	-	-	10	9.6		
12	22.3	-	-	22	22.3		
13	21.5	-	-	22	21.5		
14	0	-	-	0	0		
16	9.8	-	-	10	9.8		
23	9.6	-	-	10	9.6		
24	9.8	-	-	10	9.8		
25	7.9	-	-	8	7.9		
26	24.8	-	-	25	24.8		
27	48.4	* 40.6	-	48	44.5		
28	20.3	-	-	20	20.3		
29	10.0	-	-	10	10.0		
30	10.2	-	-	10	10.2		
31	22.1	-	-	22	22.1		
71	19.8	19.0	19.5	19	19.4	0.4	2.1
72	20.6	23.1	20.3	21	21.3	1.5	7.2
73	0	0	0	0	0	0	
74	8.8	9.0	8.8	9	8.9	0.1	1.3
75	21.3	19.6	17.9	20	19.6	1.7	8.7
76	0	0	0	0	0	0	
77	0	0	0	0	0	0	
78	8.8	8.6	8.4	9	8.6	0.2	2.3
79	8.7	8.6	8.8	9	8.7	0.1	1.2
80	9.0	8.7	8.7	9	8.8	0.2	2.0
81	8.6	8.5	8.4	9	8.5	0.1	1.2
82	8.9	8.9	8.8	9	8.9	1.0	0.7
83	8.7	8.8	8.6	9	8.7	0.1	1.2
84	0	0	0	0	0	0	
85	0	0	0	0	0	0	
86	8.5	8.7	8.5	9	8.6	0.1	1.4
87	0	0	0	0	0	0	
88	0	0	0	0	0	0	
89	0	0	0	0	0	0	
90	8.5	8.6	8.6	9	8.6	0.1	0.7
127	0	0	-	0	0	0	
128	0	0	-	0	0	0	
129	18.3	19.4	18.7	19	18.8	0.6	3.0
130	19.6	19.7	-	20	19.7	0.1	0.4

	Diazepam (1) content recorded in tablet (mg)			Reported Level	Mean	Standard Deviation	Relative Standard Deviation
Case Number	Tablet 1	Tablet 2	Tablet 3				
131	0	0	-	0	0	0	
132	0	0	-	0	0	0	
133	8.4	8.9	-	9	8.7	0.4	4.1
134	0	0	-	0	0	0	
135	0	0	-	0	0	0	
136	0	0	-	0	0	0	
137	0	0	-	0	0	0	
150	21.4	23.6	24.1	23	23.0	1.4	6.2
151	0	0	-	0	0	0	
152	0	0	-	0	0	0	
153	0	0	-	0	0	0	
154	0	0	-	0	0	0	
155	0	0	-	0	0	0	
156	10.6	10.6	9.4	10	10.2	0.7	6.8
157	0	0	-	0	0	0	
158	0	0	-	0	0	0	
159	24.4	23.2	22.1	23	23.2	1.2	5.0
160	0	0	-	0	0	0	
Actavis	10.5	10.6	10.7	10	10.6	0.1	0.9
Teva	10.6	10.5	10.6	10	10.6	0.1	0.6
MA Batch 061	10.8	10.6	10.8	10	10.7	0.1	1.1
MA Batch 064	10.1	10.6	10.5	10	10.4	0.3	2.5
Wockhardt	9.8	10.1	10.3	10	10.1	0.3	2.5

The diazepam (**1**) content within each illicit case was found to be fairly consistent, with the largest relative standard deviation of 8.7% calculated for Case 75, which had three tablets ranging from 17.9 mg – 21.3 mg of diazepam (**1**). This case was reported as containing 20 mg of diazepam (**1**) for statistical purposes. However, there were large differences between the cases, with diazepam (**1**) content ranging between 8 – 48 mg. The cases which did not produce peaks corresponding to diazepam (**1**) did generate peaks analogous to other active drug substances as can be seen in Table 5.3.

Table 5.3. Results of HPLC analysis of different active drug standards. The case numbers listed alongside indicate cases which were analysed at the same time as the drug standard and which generated the same retention time.

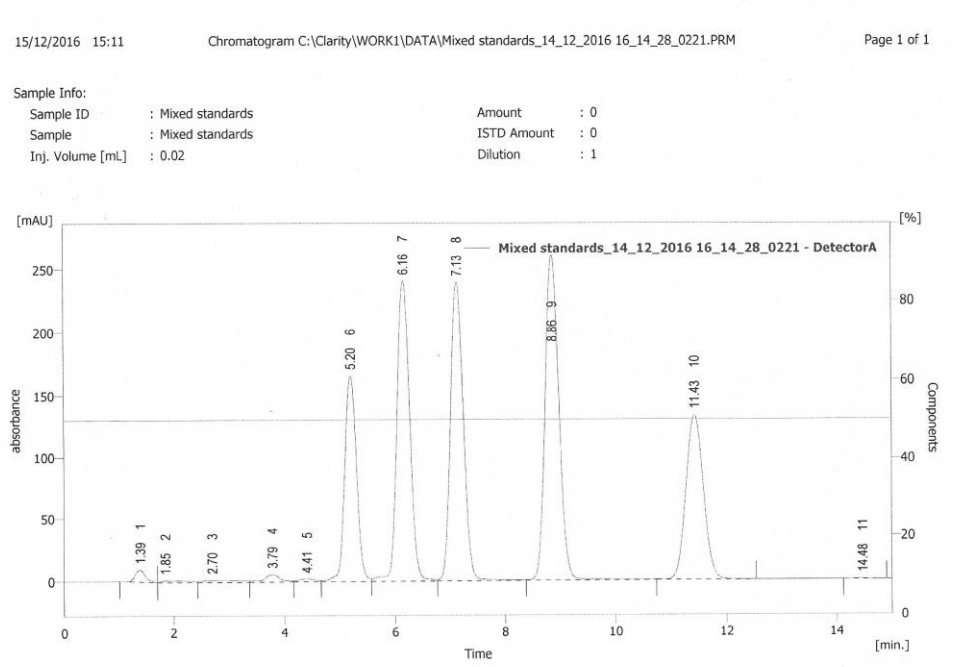
Drug standard	Retention Time (mins)	Case Numbers
Diazepam (**1**)	8.8	2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 23, 24, 25, 26, 27, 28, 29, 30, 31, 71, 72, 74, 75, 78, 79, 80, 81, 82, 83, 86, 90, 129, 130, 133, 150, 156, 159
Phenazepam (**16**)	7.1 – 7.3	77, 84, 85, 87, 88, 89, 131, 134, 137, 152
Etizolam (**7**)	6.2	73, 76, 132, 135, 151, 155, 157, 158, 160
Chlordiazepoxide (**3**)	12.1-12.2	136
Promethazine	6.6 – 6.7	127, 128, 153, 154
Acetaminophen	1.5 – 1.6	134, 152
Aspirin	1.38	73, 134, 152
Triazolam (**18**)	5.2	
Ketazolam (**10**)	11.4	

5.4.1.2 Chromatographic Separation of Active Drug Substances

Analysis of a certified ketazolam (**10**) standard by Gas Chromatography - Mass Spectrometry (GC-MS), produced a chromatographic peak with the same retention time and mass spectrum as diazepam (**1**), in this study (see Chapter 4). This meant that seized tablets which produced a peak similar to the certified standard of ketazolam (**10**) or diazepam (**1**), could not be positively identified by GC-MS alone. However, HPLC analysis of the two substances produced different retention times, enabling separation to be made between the ketazolam (**10**) and diazepam (**1**) certified standards (United Nations Office on Drugs and Crime, 2012). By running both certified standards at the beginning and end of each HPLC sequence containing samples from the seized cases, allowed comparison to be made to the

HPLC retention times. This enabled all of the seized cases in this study, which had produced chromatographic peaks similar to diazepam (**1**) and ketazolam (**10**) by GC-MS analysis, to be identified as diazepam (**1**). Each of these seized cases produced peaks with a retention time of 8.8 minutes, matching the certified diazepam (**1**) standard, instead of 11.4 minutes produced by the ketazolam standard (**10**), as shown in Table 5.3.

Similarly, distinction between triazolam (**18**) and etizolam (**7**) was clarified through HPLC analysis, thus indicating that the illicit cases tested for this project contained etizolam (**7**). A chromatogram demonstrating the retention times of various active drug substances can be seen in Figure 5.2.



Result Table (Uncal - Mixed standards_14_12_2016 16_14_28_0221 - DetectorA)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	1.393	130.524	9.740	0.7	0.9	0.20	
2	1.853	53.743	1.618	0.3	0.2	0.67	
3	2.703	80.150	1.904	0.4	0.2	0.94	
4	3.787	120.072	5.955	0.7	0.6	0.26	
5	4.407	48.906	2.334	0.3	0.2	0.38	
6	5.200	2454.481	165.728	13.6	15.6	0.23	
7	6.160	3873.396	240.624	21.4	22.7	0.25	
8	7.130	3871.842	239.333	21.4	22.5	0.26	
9	8.860	4557.109	261.479	25.2	24.6	0.27	
10	11.433	2887.638	133.085	16.0	12.5	0.34	
11	14.477	7.612	0.362	0.0	0.0	0.32	
	Total	18085.473	1062.161	100.0	100.0		

Figure 5.2. Chromatogram showing the peaks produced by five of the active drug substances. The five large peaks were identified by individual analysis to confirm retention times and from left to right, are triazolam (18), etizolam (7), phenazepam (16), diazepam (1) and ketazolam (10).

5.4.1.3 Comparison of Case 136 to the Certified Chlordiazepoxide Standard

The cases that were not identified as diazepam (**1**) were tested alongside the drug standards indicated from the GC-MS analysis. This included Case 136, which was analysed with the certified chlordiazepoxide (**3**) standard. This not only produced a peak at 12.2 minutes but was also discovered to carry over into the following blank where it generated a peak at 9.8 minutes as noted in Figure 5.3 for both illicit tablets and known standard. The reason for the two peaks is unclear but it may be due to the onset of degradation with the standard and sample being analysed at the end of a run sequence, leaving the prepared samples in slightly acidic conditions (pH5) at room temperature in the autosampler. Further investigation could have been performed on the chlordiazepoxide (**3**) standard and the samples taken from Case 136, using fresh samples just before the run and samples which had been refrigerated. The results of the further analysis would not have added more information for the aim of this study which was to separate out different potential groupings among the seized cases and was therefore not pursued.

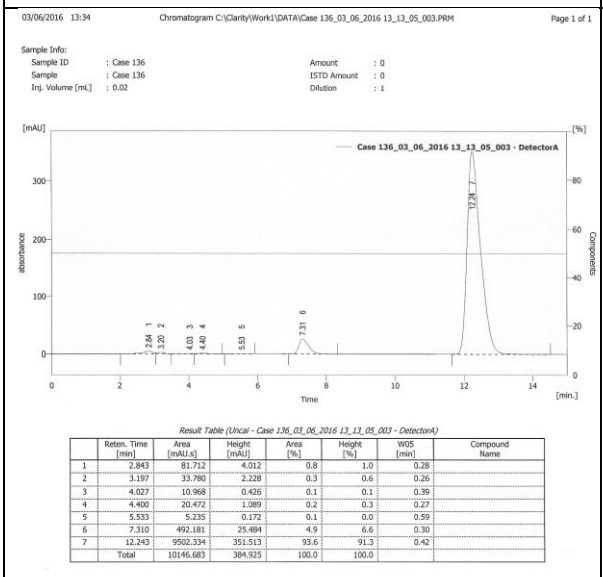
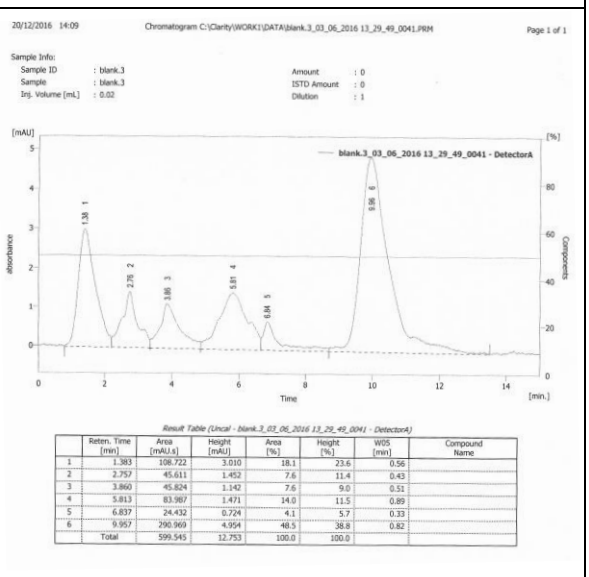
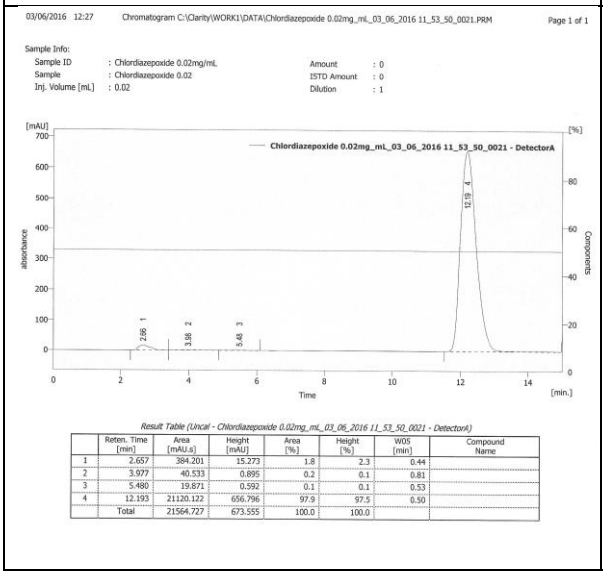
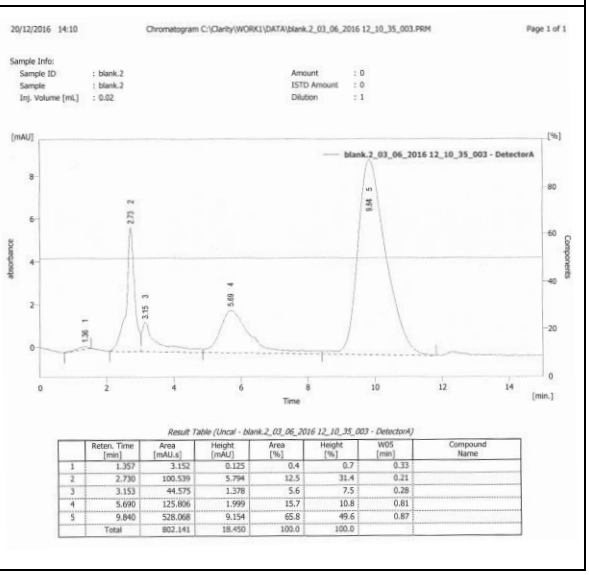
Case 136	Blank after Case 136
	
Chlordiazepoxide Standard	Blank after Chlordiazepoxide Standard
	

Figure 5.3. Case 136 and the following blank, along with the certified chlordiazepoxide (3) standard and related blank.

Interestingly, GC-MS analysis of case 136 (Chapter 4) had indicated that there may have been diazepam (**1**) present in these tablets. However, this was not corroborated by HPLC analysis, with no peaks visible at around 8.8 minutes. If diazepam (**1**) was present, it was a quantity below the LOD of 75 ng / mL.

A similar situation was discovered with Cases 127, 128, 153 and 154. These cases were analysed at the same time as the certified promethazine standard and were found to be consistent with the main peak appearing at approximately 6.6 minutes and a smaller peak carrying over into the next run at about 3.7 minutes.

The results of the HPLC analysis were added to the Venn diagram (Figure 5.4), in order to compare the new information with the previous findings. The diazepam (**1**) quantification is listed in more detail in Table 5.4. The level of diazepam (**1**) shown has been rounded to the nearest milligram and colour coded in groups of around 10 mg. The exception to the groupings, were the tablets which were analysed as containing between 9.4 – 10.6 mg, which were all reported as containing the 10 mg pharmaceutical dose to allow for errors of measurement. Anything identified as being below 8 mg was deemed too low to be classified along with the pharmaceutical tablets. No tablets were found to contain between 10 – 19 mgs of diazepam (**1**), therefore creating an acceptable differentiation point.

5.4.2 Analysis of Results

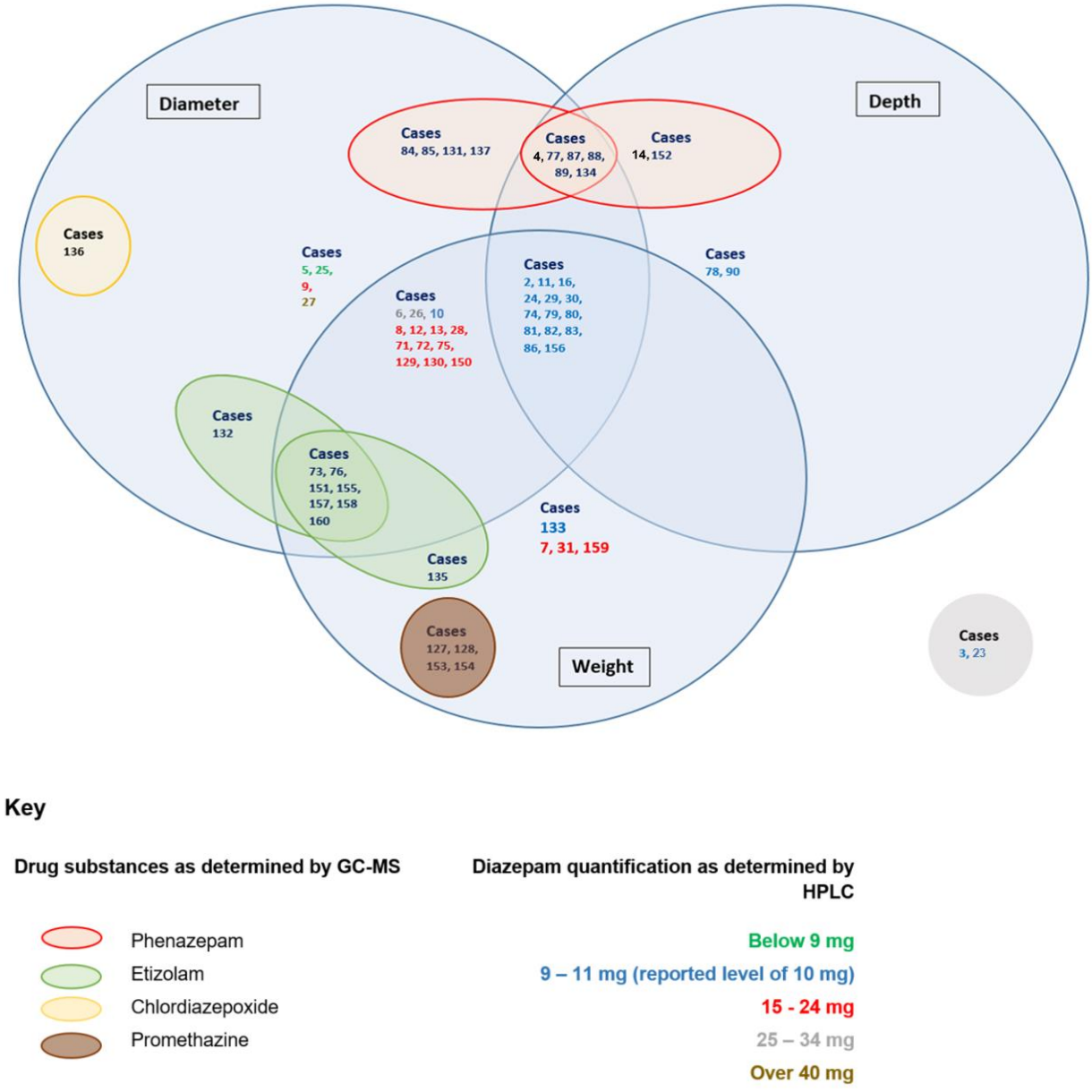


Figure 5.4. Venn diagram showing the combined results of the physical analysis, active drug substance identification through GC-MS and diazepam (1) quantification determined by HPLC. The coloured shapes identify active drug substances and colour coded case numbers relate to diazepam (1) quantification as shown in the key.

Table 5.4. Results of HPLC analysis showing the level of diazepam (1) present in the illicit cases. The case numbers have been colour coded for comparison to the Venn diagram (Figure 5.4)

Diazepam level (mg)	Case numbers
8	5, 25
9–11	2, 3, 10, 11, 16, 23, 24, 29, 30, 74, 78, 79, 80, 81, 82, 83, 86, 90, 133, 156
19	71, 129,
20	8, 28, 75, 130
21	72
22	7, 9, 12, 13, 31
23	150, 159
25	26
28	6
48	27

The Venn diagram identifies interesting clusters when combining the quantification of diazepam (**1**) with the comparison of physical measurements. Firstly, the majority of cases, which were recorded as containing a pharmaceutical level of diazepam (**1**) of between 9-11 mg appear to be comparable to the known pharmaceutical tablets in diameter, depth and weight. Indeed these cases, typed in blue, are the only ones which appear in the central area of the Venn diagram, relating to consistency with the pharmaceutical tablets. This would indicate that the illicit tablets, which do not contain the expected level of diazepam (**1**) are also not consistent in physical measurement to known pharmaceutical tablets. However, it may be that some of the seized cases, which were found to contain the reported level of 10 mg of diazepam (**1**) are also of illicit origin and further analysis is needed to further distinguish between these cases.

Interestingly, only six cases which were reported as containing 10 mg diazepam (**1**) (Cases 3, 10, 23, 78, 90 and 133), were found to vary from the pharmaceutical tablets in at least one measurement. Of the six, cases 23, 78 and 90 were imprinted with the logo MSJ and differed from the pharmaceutical tablets manufactured for the UK market in both diameter and weight (with case 23 differing in depth too). As described in Chapter 3 (Section 3.5.3.1-Tablet Diameter), MSJ markings may be consistent with the pharmaceutical company J.L. Morison Son & Jones (Ceylon) plc., which is a licensed manufacturer in other countries. However, this is not a licensed product in the UK, therefore these cases are deemed illicit. Case 3 was an isolated case marked

only with a half score on one side and is not a licensed product for the UK market. Case 133 was a single case with the marking C/DC on one side and with a plain reverse, relating to the UK licensed manufacturer Actavis. However, it measured a greater depth and diameter than the known pharmaceutical batch and had a greater relative standard deviation between the measurements recorded within the case (See Chapter 3, section 3.5.4 - Comparison of Results).

Case 10, along with Case 81 were marked CP and D/10, which is related to the pharmaceutical company Wockhardt. Although the tablets from Case 81 were consistent in measurement with the pharmaceutical tablet, Case 10 was found to vary in tablet depth

It is also worth noting that although the six cases had a reported level of 10 mg, four of them (cases 3, 78, 90 and 133) had an analytical level of below 9 mg and were rounded up to allow for errors of measurement. By rounding up the level of diazepam (**1**), these four cases reached the lower end of the pharmaceutical level.

5.4.3 Quartered Tablet

A single tablet from Case 27 was cut into quarters using a scalpel and then weighed as a whole tablet, at 192.4 mg. Each individual portion of the tablet was then re-weighed individually in order to determine the percentage of the tablet size. Every quarter of the tablet was analysed on the HPLC, as an individual sample and the results are shown in Table 5.5.

Table 5.5. The weight and diazepam (1) content found in each quarter of a single tablet taken from Case 27.

Number of Quarter	Weight (mg)	% of tablet weight	Diazepam content (mg)	% of Diazepam content	Difference in % Diazepam content
1	52.5	27.3 %	11.4	28.1%	+2.93%
2	51.8	26.9 %	11.3	27.8%	+3.34%
3	40.0	20.8%	8.2	20.2%	-2.88%
4	48.1	25.0%	9.7	23.9%	-4.40%

The results indicate that the diazepam (**1**) appears to have been fairly evenly spread throughout the tablet, with the largest quantity of diazepam (**1**) being found in the largest portion of the tablet and the second highest in the second largest etc. The difference in diazepam (**1**) content defines the variation in the amount of diazepam (**1**) found compared to the expected level in each quarter of the tablet and was calculated by using the equation ((% diazepam (**1**) content - % tablet weight) / % tablet weight)*100. However, there was a slight discrepancy, with the first two quarters showing an increase in diazepam (**1**) content compared to the weight and the smaller two having a slight loss. However, as these are illicit tablets the consistency of the batch is still unknown because consistency may vary according to its position in the hopper. Also, even if this batch was consistently produced, it may not follow that all of the other illicit cases were as well mixed. Inconsistency in mixing tablets leads to dangers of overdose, either because tablets taken are extremely high in active drug content meaning the consumer does not realise how much they have actually consumed, or because the tablets taken contain very little active drug substance, giving little pharmaceutical effect. Consequently, the consumer may take a greater quantity of tablets on the following occasion.

5.5 Conclusion

The HPLC analysis of blue tablets performed in this study was of vital importance. As the majority of benzodiazepines in the illicit market had largely been presumed to be diverted products of pharmaceutical origin (United Nations Office on Drugs and Crime, 2012), no work has previously been published on the content of seized blue ‘diazepam’ (**1**) tablets.

The results of the HPLC analysis would indicate that there is a huge variety of content within the illicit blue “diazepam” (**1**) tablet population. The tablets analysed were all believed to be being sold as 10 mg diazepam (**1**) tablets however, the level of diazepam (**1**) present within the analysed tablets was found to vary between approximately 8 mg – 48 mg in a single tablet. In terms of this project HPLC proved invaluable for distinguishing between groups of tablets containing different amounts of diazepam (**1**) content. The HPLC was also able to identify that only 20 cases out of the 65 analysed contained the pharmaceutical dose of between 9 – 11 mg of

diazepam (**1**), therefore revealing that the majority of illicit cases seized and analysed for this project were illicit. This means that only 31% of the cases had the potential to be pharmaceutically manufactured diazepam (**1**) tablets produced for the UK market, which had been diverted into the illegal supply chain. This may reflect a change in the illicit market from diverted pharmaceutical tablets (Advisory Council on the Misuse of Drugs, 2016) to illicitly manufactured products, with greater access to knowledge and equipment for clandestine operations (See Chapter 4, section 4.4.5 – Lubricants).

In addition, the variety of active substances found including phenazepam (**16**) with a potency five times greater than diazepam (**1**) and etizolam (**7**) with a potency ten times higher reflect the variation and dangers within the illicit market. The situation would be magnified if multiple tablets are consumed within a short time span or in conjunction with other drugs and / or alcohol, which could have potentially serious consequences. Therefore the results of this analysis are extremely important and provide invaluable information for both police and medical services.

Chapter 6. Differential Scanning Calorimetry

6.1 Chapter Summary

Differential scanning calorimetry was tested as a way to explore thermal differences between batches of tablets. A large fraction of a pharmaceutical diazepam (**1**) tablet is comprised of excipients, with only 10 mg of active drug substance present in the samples tested for this project. Therefore, thermal events detected by DSC have the potential of providing valuable information on the excipient content of the illicit tablets by comparison to thermograms produced by known pharmaceutical preparations. The technique was also explored as a way to differentiate between different batches of seized tablets.

6.2 Introduction

6.2.1 Types of Differential Scanning Calorimeter

Differential Scanning Calorimetry (DSC) is a well-established analytical technique that enjoys widespread use in both polymer characterisation and pharmaceutical development. However, it is not commonly used for the forensic examination of illicit tablets.

There are two kinds of DSC, a power compensation system and a heat flux system. The former uses two heaters, one for the sample and the other for the reference pan. The heaters utilise exactly the same temperature programme and it is the difference in power used to keep the temperature the same, which is measured. The heat flux DSC, which was employed in this study, uses a furnace to heat the sample and reference together and uses a thermocouple to measure the difference in the heat flow between the two pans. The heat flow is affected by the thermal changes occurring in the sample (Craig and Reading, 2006).

Changes to the heat capacity of a sample during a heating or cooling cycle can be used as an indication of the chemical reactions or phase changes, both of which are characteristic of the sample being tested. Phase changes that can be detected with DSC include dehydration, de-solvation, recrystallization, melting and degradation.

6.2.2 Pharmaceutical Use

6.2.2.1 Polymorphism in Solid Substances

DSC is used extensively in the pharmaceutical industry, to test for the presence of polymorphic drug forms by measurement of melting/recrystallization behaviour and it can also be used to quantify the degree of crystallinity in samples. The physicochemical properties of drugs and excipients are affected by the occurrence of polymorphism and this can influence the dissolution and solubility of tablets and the bioavailability of the drug substance itself (Aulton, 2007a). Tablet storage, shelf-life and drug stability are all governed by the precise crystalline nature of tablet components and DSC is used to determine these inherent properties. A study in 2008 explored the importance of understanding the thermal behaviour of polymorphs in relation to using high speed DSC in order to prevent concurrent recrystallization. The benefit of this was that it could restrict kinetically controlled processes while allowing the thermally activated events to take place (Mcgregor and Bines, 2008). In simple terms, fast heating does not give enough time for metastable polymorphs (upon melting) to recrystallize into more stable forms. This simplifies the results produced by eliminating exothermic events caused by so-called 'solid state transitions', i.e. one solid polymorph changing to a more stable solid polymorph through a recrystallization process.

Polymorphism can also complicate any quantification performed by DSC. For example, if diazepam (**1**) is present in the tablets, an endothermic peak will be produced by the breaking of ionic interactions and/or secondary bonds such as hydrogen bonds and Van der Waals forces, as the drug substance melts. However, when amorphous forms of the drug are present, the melting temperature of the diazepam (**1**) may vary. In these terms, 'amorphous' equates with 'non-crystalline'. Semi-crystalline materials have a mixture of crystalline and non-crystalline parts and a broader onset to the crystalline melt is commonly encountered. A slight depression (lowering) of the peak maximum may also be apparent.

A study by Bruni *et al.*, (2012) explored methods of quantification of amorphous and crystalline fractions. The paper notes that the crystalline structures are more stable

but sometimes the amorphous form of the active drug substance is preferred as it improves bioavailability. A large proportion of the pharmaceutically manufactured tablets are comprised of lactose, which contains both crystalline and amorphous forms (Rowe, Sheskey and Weller, 2003). As a result, an exothermic peak can be seen on the thermograms when the particles anneal.

The ***α***-lactose monohydrate present in pharmaceutical tablets, has water molecules within its structure along with additional water molecules that are adsorbed onto the surface. Therefore, as the sample is gradually heated in the DSC, the initial falling line on the thermogram relates to desorption of the moisture. This is followed by the ***α***-lactose monohydrate dehydration and then its melt. It has been recorded that the starting point for lactose to become anhydrous is 120 ^{o}C, producing an endothermic peak at 150 ^{o}C and it begins to melt at 201 ^{o}C. The resulting peak produced by the melt varies according to the size of the particles (Rowe, Sheskey and Weller, 2003). The β-lactose melt takes place after this at 224 ^{o}C (Figure 6.1).

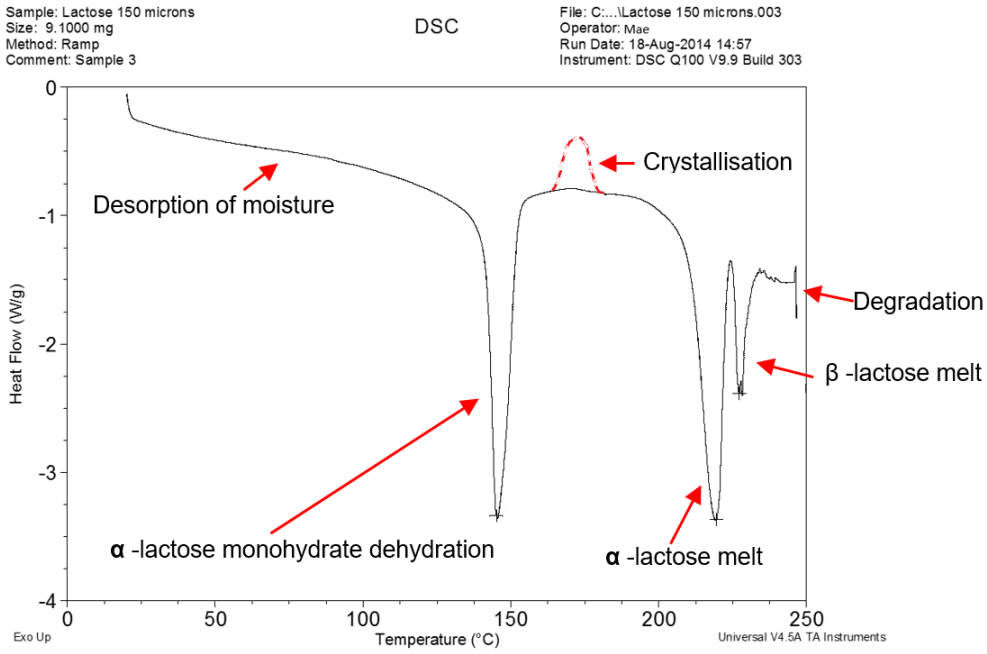


Figure 6.1. DSC thermogram of lactose identifying the thermal events. There was very little crystallisation of this sample, therefore the peak was drawn in for demonstration.

The thermal history of a tablet can also have a significant impact on the shape of the thermogram produced. This is particularly true of semi-crystalline substances which

are strongly affected by the length of time taken to heat or cool the sample. Basically, the more time taken to cool, the more likely it is to have settled into a more ordered crystalline structure (Craig and Reading, 2006).

6.2.2.2 Compatibility Studies

Results produced by DSC can be problematic to interpret if multiple components are present in the tablets. When only one pure substance is present, such as diazepam (**1**), a clear single peak is shown (Figure 6.2). However, compatibility studies are another important investigation performed by DSC within the pharmaceutical industry, aimed at ensuring the safe use and stability of the formulation.

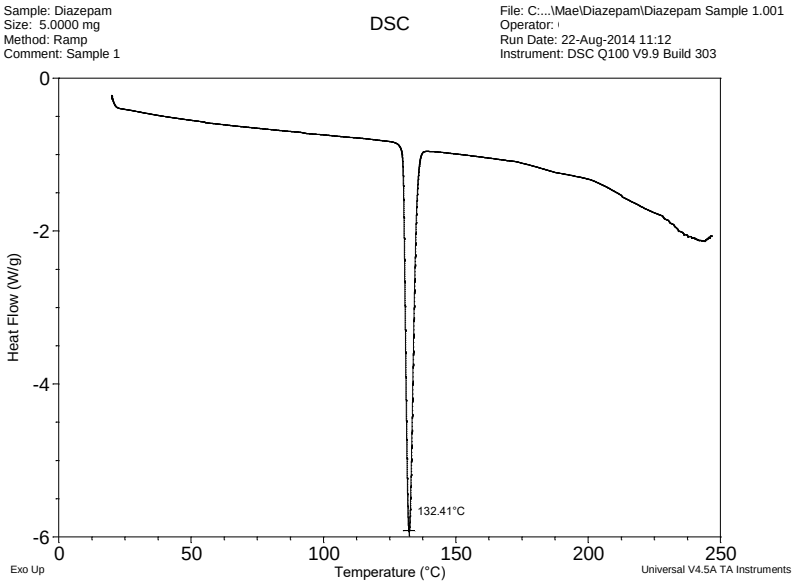


Figure 6.2. DSC trace produced by Diazepam (1)

Tablets usually contain a variety of excipients, each with a different function but which also provide bulk to the tablet. All of these added components produce their own trace by DSC, as well as further reactions derived from the combined ingredients interacting with each other. In addition, when low levels of constituents are present, including the active drug substance, it can be overwhelmed and hidden on the thermogram because of the large quantity of other excipients. As illicit tablets contain an indefinite variety of unknown substances in unspecified quantities, the results can therefore be very difficult to interpret.

A recent study investigated the compatibility of diazepam (**1**) with various tablet excipients using thermal analysis. No incompatibilities between the substances were found but DSC did indicate that an interaction had taken place between the diazepam (**1**) and colloidal silicon dioxide by demonstrating a lower enthalpy value (Matos *et al.,* 2017). Colloidal silicon dioxide is present in tablets produced by Sovereign Medical (Medicines and Healthcare Products Regulatory Agency, 2016b) and consequently pharmaceutical tablets produced by this company may show slightly smaller thermal peaks compared to other pharmaceutical tablets.

6.2.3 Forensic Use of DSC

Forensic interest has recently been growing into the potential use of DSC. A study in 2015 used DSC to investigate amphetamine type stimulants without the potential interference caused by creating artefacts through pre-treatment of samples (Boumrah *et al.,* 2015). Research performed into illicit diazepam (**1**) tablets, suggests that DSC may be a powerful tool for identifying similarities and differences between cases (Bibi *et al.,* 2015).

One of the benefits of using DSC for forensic analysis is that the excipients within the tablets and their interactions can be investigated. Many of the more widely used methods of analysing illicit tablets concentrate on determination of the active drug substance. However, as the amount of diazepam (**1**) present in the tablets is so small, DSC is heavily influenced by the excipient content, thus it produces a chemical profile that reflects primarily the thermal character of the excipients.

6.3 Overview of DSC analysis

A selection of known tablet excipients and a variety of different grades of lactose were analysed by DSC. In addition, different brands of known pharmaceutical tablets were also analysed. The resulting thermograms were visually compared to the illicit cases. Statistical analysis was also performed on the data and the results can be found in Chapters 7 and 8.

Multiple samples were tested from the same tablets to ensure repeatability and consistency within individual tablets. Repetitions were also tested using multiple tablets from the same case.

The aim of the analysis was to investigate if it would be possible to create a chemical profile of the illicit cases that could give an indication of the constituents present within the tablets, by comparison to thermographic peaks of common excipients. In addition, by producing thermograms which were visually very similar this may highlight chemical similarities between cases that had not previously been realised. For example, if an illicit manufacturer sticks to a formula that produces successful tablets but just changes the active ingredient, then the thermogram is likely to be very similar, particularly if the active drug substance in only added in small quantities. It was also intended that comparisons with the known pharmaceutical tablets should help to denote a potential origin of the illicit cases. Likewise, seized cases which produced DSC thermograms similar to those of known pharmaceutical tablets could support the previous analysis regarding physical characteristics and diazepam (**1**) content in identifying potential cases or pharmaceutical origin.

6.4 Experimental

6.4.1 Instrumentation

Differential Scanning Calorimeter (DSC) thermograms were produced on a TA Instruments Q100 Differential Scanning Calorimeter with RCS90 cooling system, using Thermal Advantage software. Samples were analysed in standard aluminium

pans set with a starting temperature of 20 ^{o}C. The heating rate was ramped up at 10 ^{o}C per minute to a final temperature of 250 ^{o}C. The temperature was calibrated against indium, with a melting point of 156.60 ^{o}C, as shown in Figure 6.3.

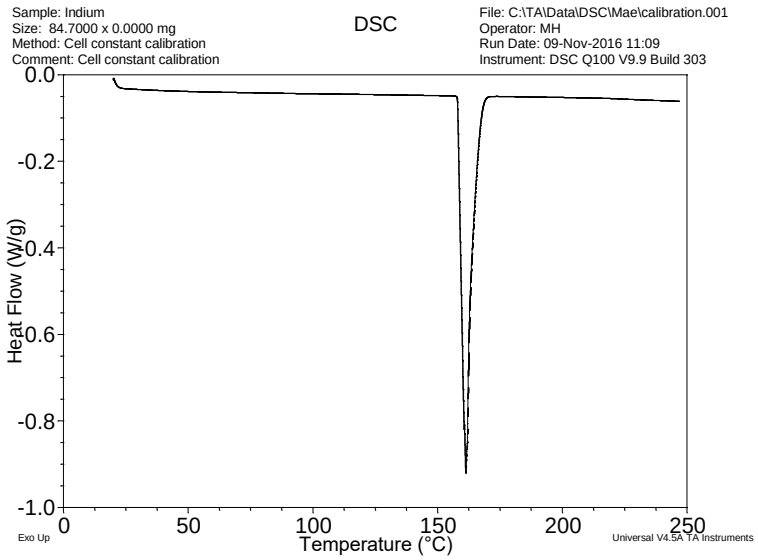


Figure 6.3. Calibration of DSC with Indium.

6.4.2 Materials used in DSC Analysis

Illicit blue tablets were provided by Police Scotland. 10 mg diazepam (**1**) pharmaceutical tablets were supplied by Actavis UK Ltd., Barnstaple; Teva Pharmaceutical Industries, UK; and Wockhardt UK Ltd., Wrexham. Two different batches of 10 mg diazepam (**1**) pharmaceutical tablets were provided M&A Pharmachem Ltd., Bolton. Certified standards of diazepam (**1**) were obtained from Sigma Aldrich and Cow and Gate Follow on Baby Milk was purchased from Asda, Aberdeen. Standard DSC pans (900779.901) and standard lids (900786.901) were supplied by TA Instruments Ltd, Crawley.

6.4.3 Methodology used in DSC Analysis

Samples for DSC were prepared by crushing a tablet with a clean mortar and pestle. A small quantity between 5 – 10 mg of the powdered sample was place in a sealed aluminium pan and heated alongside an empty reference pan, in the DSC at the predetermined rate. The temperature difference between the pans was recorded.

(Although the samples could range between 5 – 10 mg in weight, the initial analysis aimed at using a consistent sample size of 9 mg, in order to avoid unnecessary discrepancies).

Samples were prepared from common tablet excipients including polyvinylpyrrolidone (PVP), also known as Povidone. This is a synthetic polymer that is used as a binder and is present in tablets manufactured by MA (MA Pharmachem Ltd, 2011), and Wockhardt (Wockhardt UK, 2015). Other excipients analysed were magnesium stearate, which is a lubricant present in most pharmaceutically manufactured diazepam (**1**) tablets and *Emcompress*™. *Emcompress*™ is the trading name for calcium hydrogen phosphate dihydrate. This is a commonly used filler and diluent used in the pharmaceutical industry (Rowe, Sheskey and Weller, 2003). Interestingly, it is not normally used in diazepam (**1**) tablets that are produced for the UK market. In addition, various grades of lactose were run using DSC, in order to assess if a difference in grade would affect the resulting thermogram. Lactose is a tablet filler or diluent used in all pharmaceutically manufactured diazepam (**1**) tablets in the UK.

Samples were also prepared and run from each of the batches of known pharmaceutical tablets for comparative purposes. This included multiple runs of up to three samples produced from the same powdered tablet and up to eight samples prepared from different tablets within the same batch.

One tablet was taken and prepared from each of the illicit cases. Depending on the number of tablets available, more than one sample was occasionally analysed from the same tablet, or multiple tablets were investigated from the same batch. The repeats were performed on random tablets and consistency of the results was evident.

Repeats of some of the samples were performed between 9-10 months later and some of the pharmaceutical cases were repeated after 4 months.

Whole tablets were placed in small plastic bags and powdered tablets were contained in vials for storage. These were not kept in a humidity or temperature controlled environment. Storage of the tablets before seizure was also unknown.

6.4.4 Repeatability of the Analysis

Eight separate pharmaceutical tablets from MA Pharmachem were prepared and analysed. Consistent results were demonstrated, as shown in Figure 6.4.

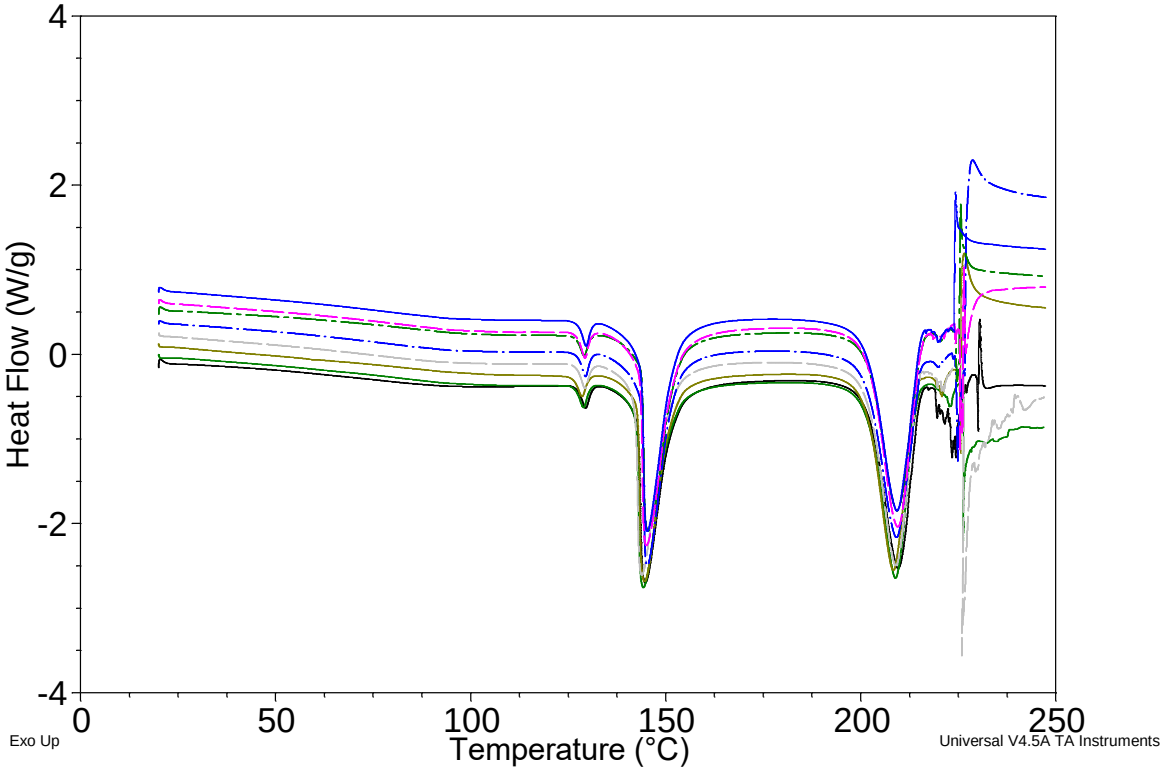


Figure 6.4. Comparison of thermograms produced by eight different pharmaceutical tablets supplied by MA Pharmachem. Differences occurring after 220 ºC were the result of sample degradation and provided no relevant information for this project.

6.4.5 Temperature Range Analysed

The temperatures analysed for samples ranged between 100 – 220 ºC. Although the thermograms were recorded from 22 ºC, many of the profiles indicate a gentle slope reflecting a decrease in heat flow up to 100 ºC. This is likely to be a result of solvent and water loss according to storage rather than being directly characteristic of the drug substances and excipients present in the tablet.

Similarly, many of the samples analysed began to degrade at 220 ºC as demonstrated in Figure 6.4 and by the thermogram produced by the Actavis tablet shown in Figure 6.8. This meant that although the β-lactose melt had been recorded at 224 ºC (Figure 6.1), this was not always consistent due to falling within the area of degradation.

It was therefore decided that these areas would not be included for the analysis or for the data collection used in later statistical analysis. However, a great deal of data was gained from the thermograms and data produced within the 100 – 220 ºC range.

6.5 Results and Discussion

6.5.1 Common Excipients used in Pharmaceutical Tablets

No surprisingly, the thermograms produced by the excipients demonstrated considerable differences in profile (Figure 6.5).

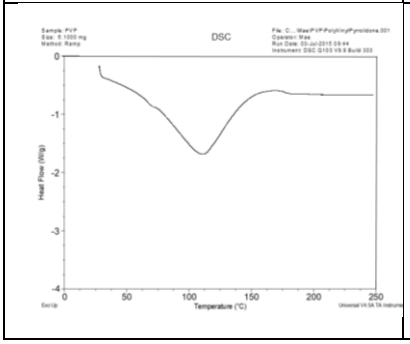
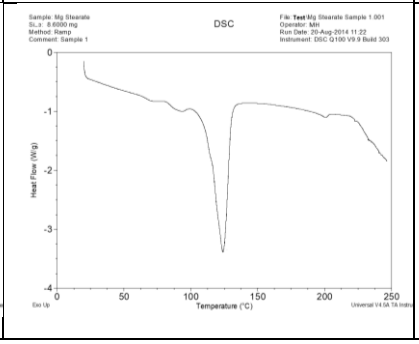
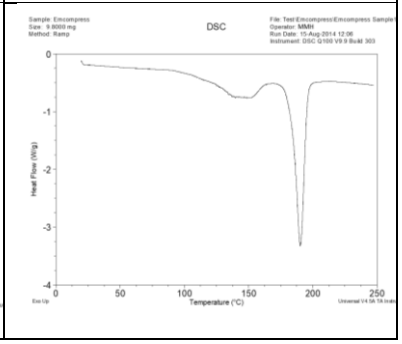
PolyVinylPyrrolidone (Povidone)	Magnesium stearate	Emcompress™
		

Figure 6.5. Thermograms of three tablet excipients (Povidone, Mg Stearate and *Emcompress*™) at a heating rate of 10 °C / min.

However, there was less of a difference between the different varieties of lactose (Figure 6.6). Although they are different grades, they are effectively the same substance and therefore all lactose samples exhibited endothermic peaks at about the same temperature for the dehydration and melt of the ***α***-lactose monohydrate.

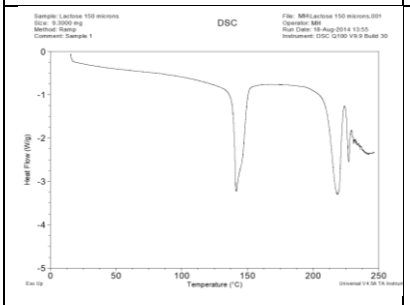
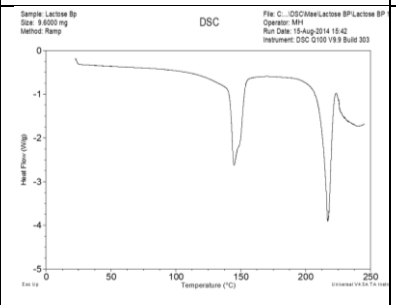
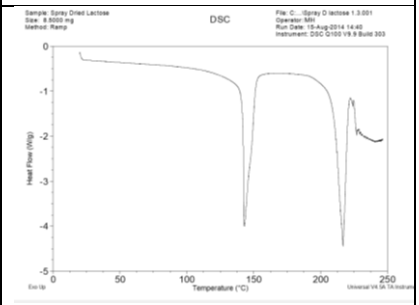
150 micron lactose	Lactose Bp	Spray dried lactose
		

Figure 6.6. Thermograms of different lactose grades showing similarity in endothermic peaks.

6.5.2 Certified Drug Standards Tested for Comparative Purposes

Most of the drug substances analysed produced different thermograms by DSC (Figure 6.7) although the melting points of the phenazepam (**16**) and nitrazepam (**13**) were similar. Clonazepam (**4**) also demonstrated a high melting point but it appeared closer to 240 ^{o}C, than the other two. Although the analytical grade of the drug substances produce thermograms with distinct melting points, it is important to be aware that polymorphic forms or impurities can affect the melting point. Therefore the drug substance suggested by comparison is not conclusive for identification purposes. Illicit tablets in particular, contain a variety of unknown substances which interact with the active drug substance. Synthesis of drug substances involves the use of different organic solvents to recrystallize the drug during recovery and purification. Many drugs can form solvates and these have their own specific thermal behaviour which normally involves the loss of solvent to form a metastable desolvate, followed by subsequent recrystallization into a more stable polymorphic form. Also, the origin of the drug substance itself is unspecified therefore the level of purity is unknown.

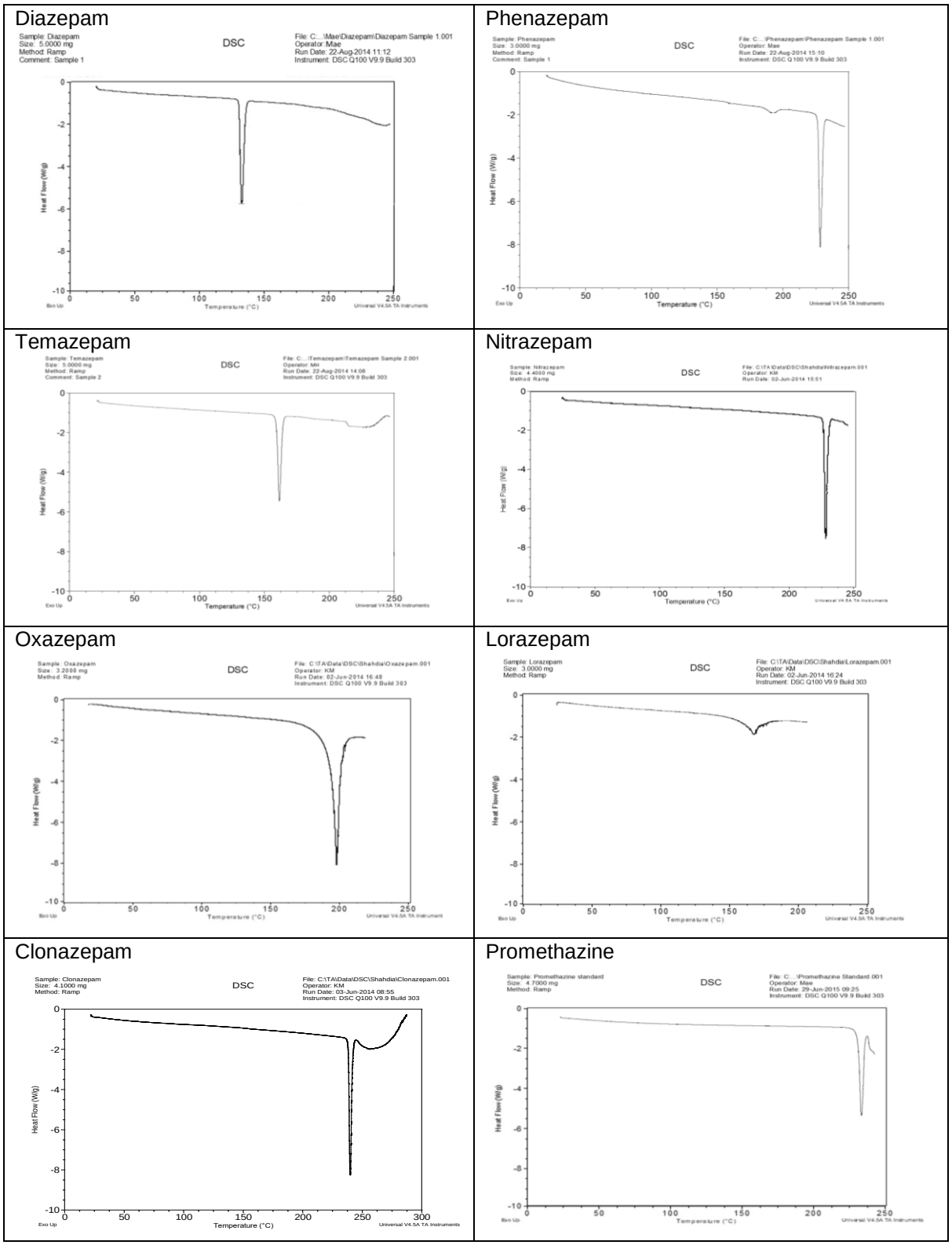


Figure 6.7. Thermograms produced by a selection of benzodiazepines using a single heating cycle at 10 °C / min from 20 to 200 – 250 °C. Thermograms of nitrazepam (13), oxazepam (15), lorazepam (11) and clonazepam (4) courtesy of Robert Gordon University.

6.5.3 Pharmaceutical Tablets Analysed by DSC

The results of DSC traces on the known pharmaceutical tablets proved interesting. All of the thermograms appeared to be dominated by lactose, which was one of the known ingredients. Therefore the expected dehydration and melt of the ***α***-lactose monohydrate was present. Occasionally a peak could be seen which could be indicative of the β-lactose melt at 224 ^{o}C however, this could be subjective as it was either close to or within the area of degradation which began at 220 ^{o}C.

The majority of the pharmaceutical cases produced a very similar thermogram, as demonstrated by the MA trace shown in Figure 6.8. The thermogram generated by the Actavis tablet exhibited some differences, however. Firstly the endothermic peak produced by the diazepam (**1**) melt at 130 ^{o}C was barely visible. Only the very tip can be seen on the trace shown in Figure 6.8. In this case, it is largely hidden by the ***α***-lactose dehydration. Another endothermic peak is also visible at just above 58 ^{o}C. Earlier work performed at Robert Gordon University identified this peak as stearic acid (Bibi *et al.,* 2015). This is a lubricant which is listed by Actavis as being in their formulation (Actavis, 2014).

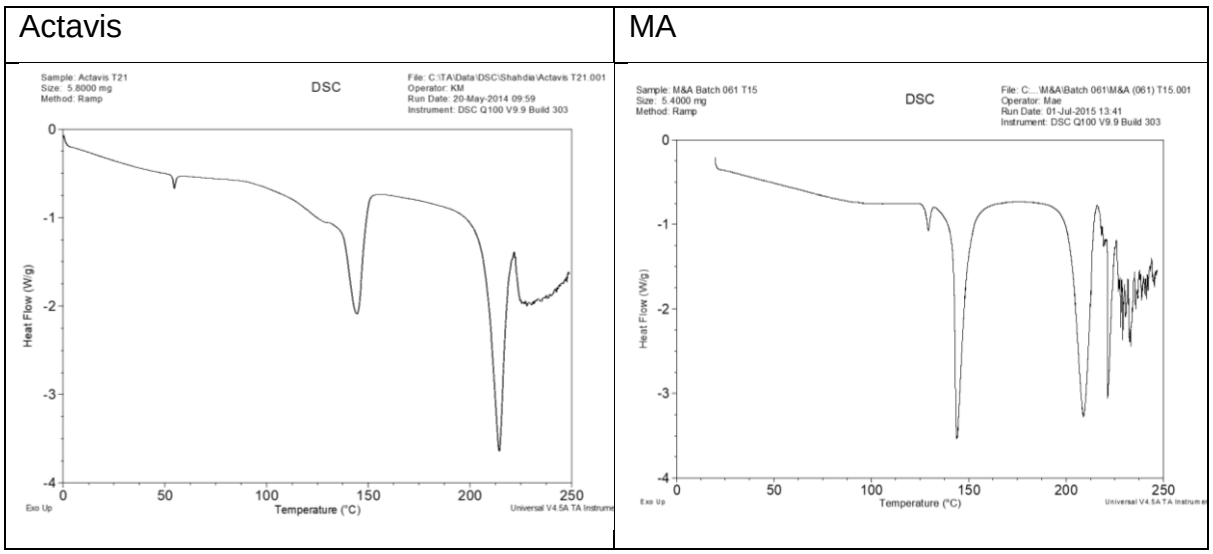


Figure 6.8. Thermograms showing the differences between DSC profiles produced by the Actavis tablet compared to the MA tablet. The pharmaceutical companies Teva and Wockhardt manufactured tablets generated thermograms that were almost identical to that of the MA tablet. Thermogram of Actavis tablet courtesy of Robert Gordon University.

The indication of diazepam (**1**) concentration within the tablets was also detected in the pharmaceutical tablets. Although Roche do not produce diazepam (**1**) tablets for the UK market, the tablets were originally treated as being of pharmaceutical origin and the data produced was consistent with results from the other legitimate manufacturers. By analysing two Roche tablets, one containing 2 mg and the other 10 mg of diazepam (**1**), a difference in the height of the peak produced by the diazepam (**1**) melt emerged (Figure 6.9).

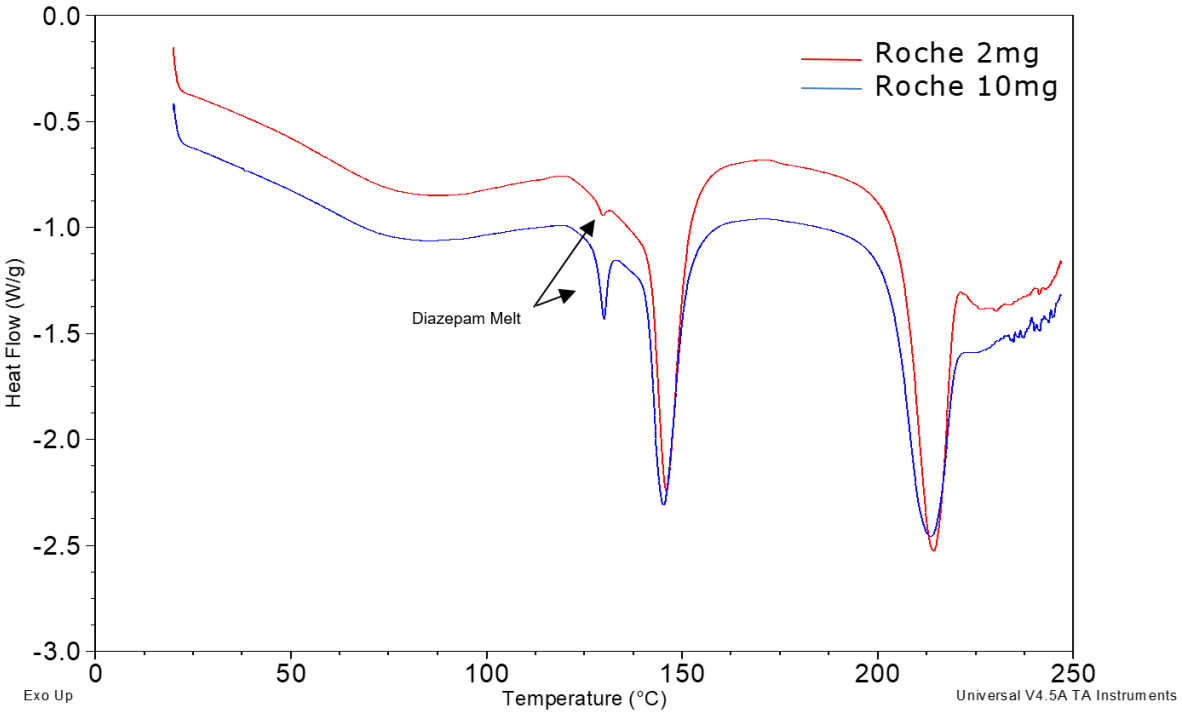
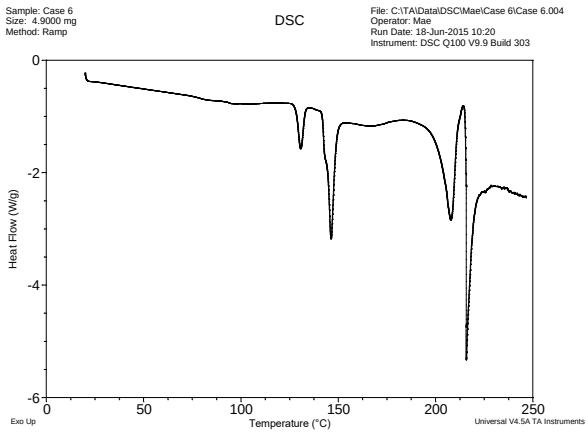
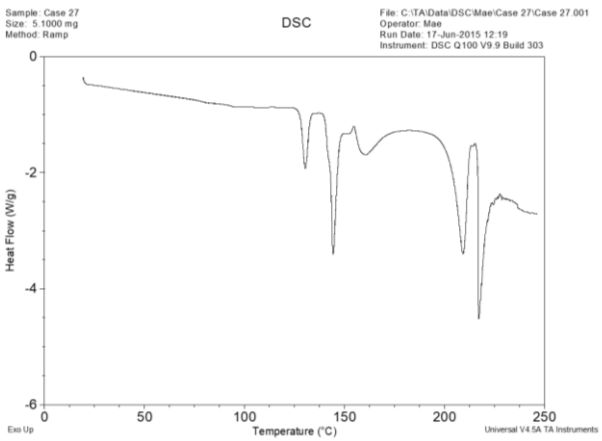


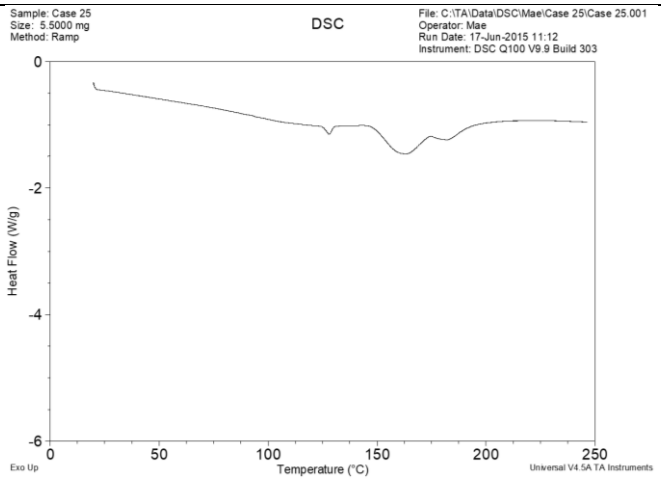
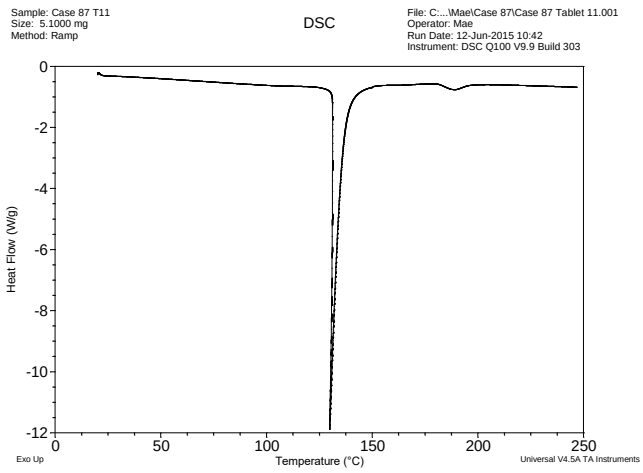
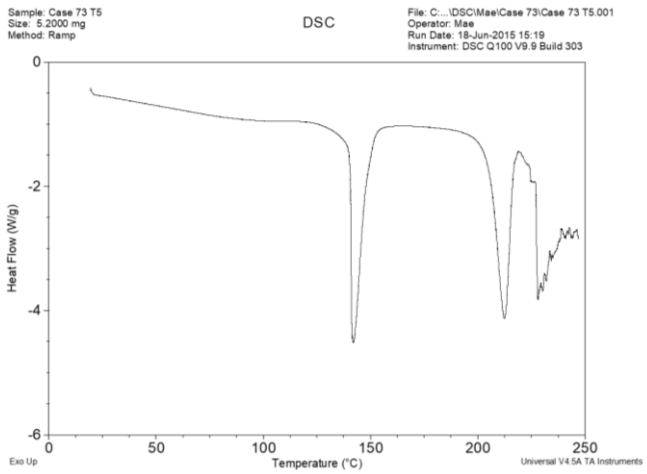
Figure 6.9. Thermogram showing the difference in peak size produced by melting tablets with different amounts of diazepam (1).

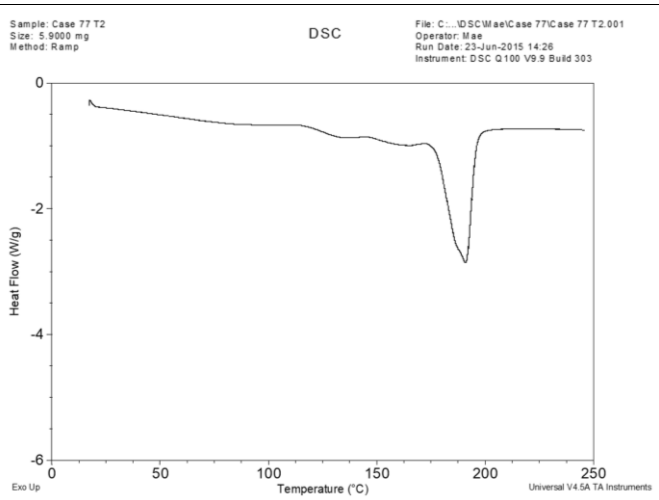
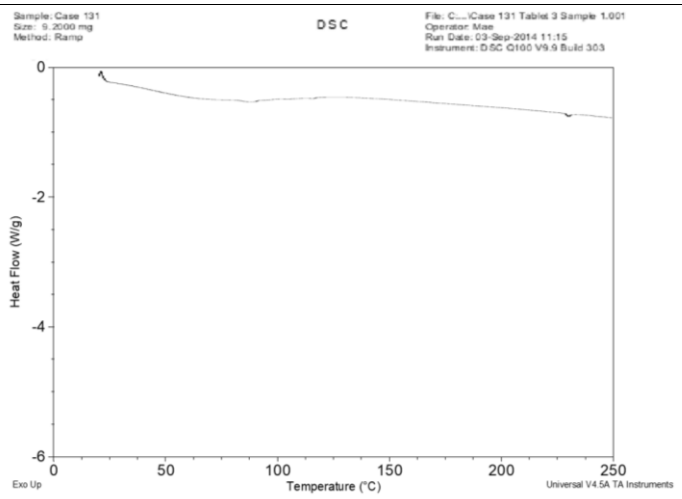
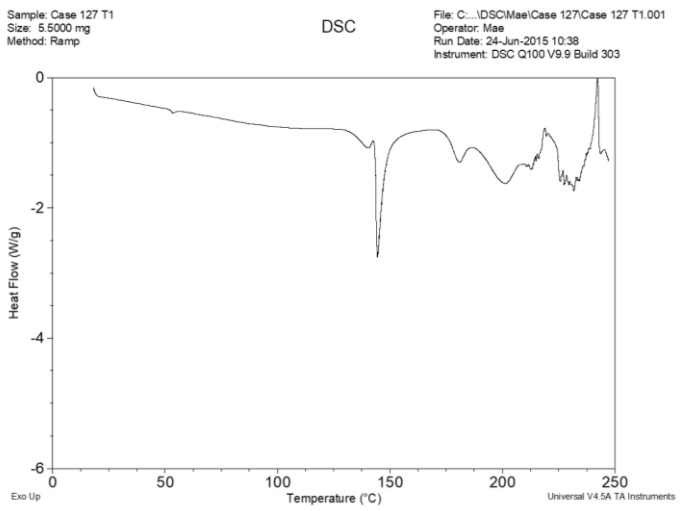
6.5.4 Illicit Cases Analysed by DSC

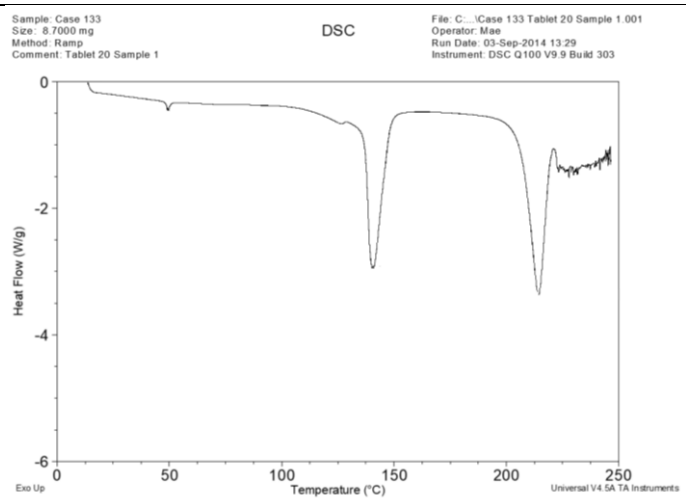
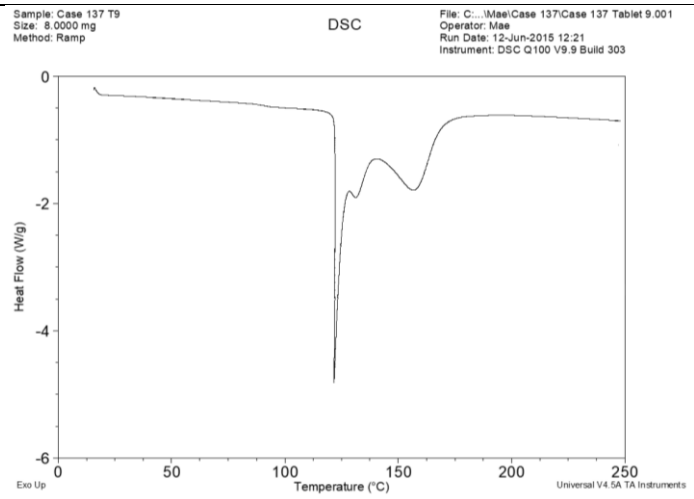
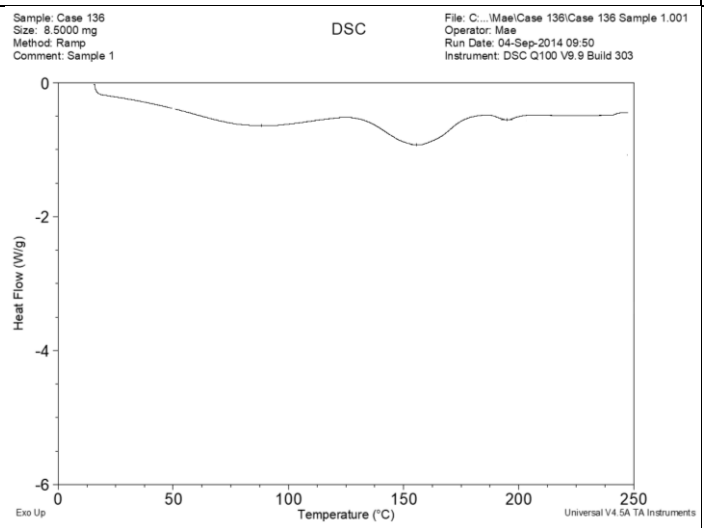
At least one tablet from each case was used for DSC analysis in order to assess any potential links between the cases. It was discovered that the 65 illicit cases could be visually grouped into eleven potential sub-groups based on the thermograms produced. The details of the groupings can be seen in Table 6.1. This table lists all of the cases that generated similar thermograms, along with an example of the trace. This allowed for minor differences, such as in the area of degradation and crystallisation.

Table 6.1. Grouping of the thermograms produced by the various cases.

Group	Thermogram	Case Numbers
Group 1		2, 6, 7, 8, 9, 10, 11, 12,13, 16, 23, 24, 26, 28,29, 30, 31, 71, 72, 74,75, 78, 79, 80, 81, 82,83, 86, 90, 129, 130, 150, 156, 159
Group 2		27

Group	Thermogram	Case Numbers
Group 3		3, 5, 25
Group 4		4, 14, 87, 88, 152
Group 5		73, 76, 132, 135, 151, 155, 157, 158, 160,

Group	Thermogram	Case Numbers
Group 6		77, 89, 134
Group 7		84, 85, 131
Group 8		127, 128, 153, 154

Group	Thermogram	Case Numbers
Group 9		133
Group 10		137
Group 11		136

Group 1

The cases listed in Group 1 include, with a few notable exceptions, the majority of illicit cases which were found to contain diazepam (**1**). The fact that these tablets have been linked together is somewhat subjective because they were found to contain varying amounts of diazepam (**1**), ranging from 9 mg in Case 74 to 28 mg in Case 6. The difference in concentration is not immediately noticeable in the thermograms, which are largely dominated by the lactose.

Group 2

Interestingly, Case 27, which has been listed as Group 2, also produced a fairly similar trace to Group 1. The difference with this profile was the extra endothermic peak after the dehydration of the ***α***-lactose monohydrate, at 160 ^{o}C. Tablets in this case bore the same markings as Case 6, but were found to contain approximately 48 mg of diazepam (**1**) by HPLC analysis. Comparison of the ratio of diazepam (**1**) to lactose dehydration in Groups 1 and 2 indicates an increased level of diazepam (**1**) in Group 2.

Group 3

The cases in Group 3 were an interesting mix. Cases 5 and 25 had been recorded in the visual clustering as being slightly chalky in appearance and were found to contain approximately 8 mg of diazepam (**1**) by HPLC analysis. Ageing and storage of tablets may have promoted the degradation of the drug substance and appearance of these tablets and therefore they could not have been conclusively deemed as being illicitly manufactured. However, DSC analysis indicated that the thermogram produced by Cases 5 and 25 were different to the pharmaceutically manufactured tablets.

Case 3 appeared very different visually to Cases 5 and 25, having different markings on the tablets and being convex in shape. However, tablets in this case were also found to contain a slightly lower amount of diazepam (**1**) at approximately 9 mg. Whether theses tablets had suffered through storage or were produced from a completely different formulation is uncertain.

Group 4

The cases in Group 4 were all found to contain phenazepam (**16**). Although the single endothermic peak is closer to the temperature indicated for the melt of diazepam (**1**), it may be that the form of the phenazepam (**16**) has altered its melting point. However, using HPLC, it was discovered that the level of phenazepam (**16**) present in the tablets was very small so, it is unlikely that this would produce such a strong peak. Considering the magnitude of the heat transfer (almost 20 W/g), the precise temperature at which it occurred and the very sharp nature of the event, a single substance which is most probably an excipient, is dominating this tablet sample. Without extra corroborating data such as hot stage microscope or thermal gravimetric analysis it is not possible to state the nature of the event.

Group 5

The Group 5 tablets appear to have a very similar formulation to the pharmaceutical diazepam (**1**) tablets. The only difference is that there is no melt for the diazepam (**1**). GC-MS and HPLC revealed that these tablets contained etizolam (**7**) and in fact, every case that was found to contain etizolam (**7**) was in this group. It appears that the main constituent in them is lactose and the small amount of drug substance present was not obvious in the form of an endothermic event. The tablets had a variety of logos and there were differences in their shades of blue. Although well made in appearance, the fact that etizolam (**7**) is not present in tablets produced for the UK market identifies these cases as illicit.

Group 6

The three cases in Group 6 were found to contain phenazepam (**16**) and once more, the small amount of active drug substance present is not clearly visible and may be hidden behind the strong trace produced by the excipients. In these cases, the endotherm with an onset of 175 ^{o}C is consistent with the dehydration generated by *Emcompress*^{TM}, as demonstrated in Figure 6.5.

Group 7

Further analysis of the cases in Group 7 indicated that these also contained phenazepam (**16**), though clearly with a different formulation to those in either Group 4 or 6. It appears that the thermogram is shaped by the excipients. Although the endothermic event at 225 ºC is in the area of degradation, shown in greater detail in Figure 6.10, this small peak appears clearly on all three thermograms. It is also close to the melt of phenazepam (**16**) indicated in DSC analysis of the drug substance, as seen in Figure 6.7.

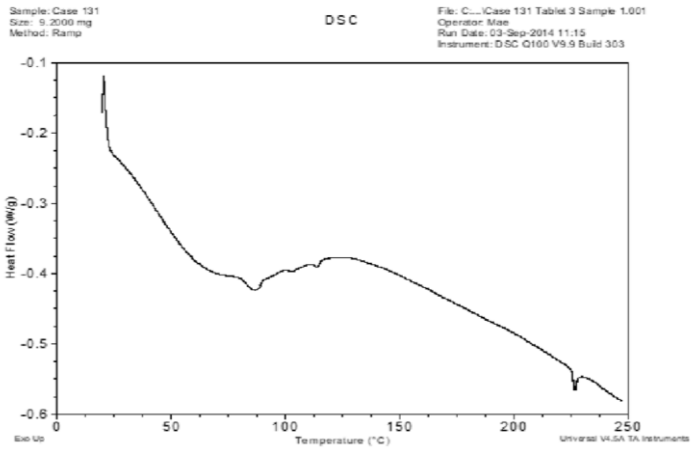


Figure 6.10. Closer detail of thermogram produced by Group 7 cases.

Case 85 was the only one of the three Group 7 cases, which generated an additional small endothermic peak at 165 ºC. Whether this was hidden in the remaining cases by the steep slope produced by melting is uncertain. It may also be that whatever this constituent was, could have been present in a slightly larger concentration in Case 85.

Group 8

The Group 8 tablets included all of the cases, which GC-MS identified as containing promethazine. Unfortunately, there is too much sample degradation occurring to see anything meaningful above 200 °C in this group.

Group 9

The thermogram produced by Case 133, which is listed as Group 9 was consistent with that produced by the pharmaceutical tablets manufactured by Actavis. There was only a small indication of the diazepam (**1**) melt at 130 ^{o}C, which was obscured by the dehydration of the ***α***-lactose monohydrate. The endothermic peak at 50 ^{o}C, relating to stearic acid was also visible. In addition, the tablet markings were consistent with those used by Actavis.

Groups 10 and 11

The remaining two groups contained only one isolated case each. Group 10, which consisted of only Case 137 was found by GC-MS analysis, to contain phenazepam (**16**) and caffeine and Case 136 (Group 11) was the only case that was identified as containing chlordiazepoxide (**3**) by GC-MS. As the level of phenazepam (**16**) in Case 137 was small and the melting point of caffeine and chlordiazepoxide (**3**) is well in excess of 200 °C, the two thermograms appear to be dominated by the unknown excipients.

6.5.5 Effects of Excipients

Although, it is not possible to identify excipients through this analysis because there are so many influencing factors and interactions that must be considered, some similarities do appear. For example, the thermogram produced by Case 136 is similar to a trace produced by a large concentration of lactose. As a result of this, it was decided to run a sample of 'Cow & Gate Growing Up Milk, age 1-2 years'. As it consists mainly of lactose, this produced a result that was similar in shape to Case 136 (Figure 6.11).

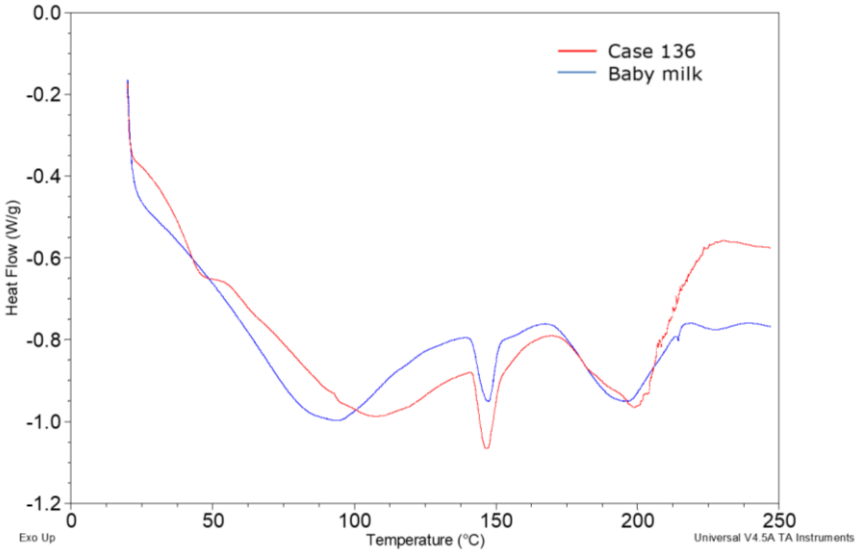


Figure 6.11. Thermogram from Case 136 overlaid with Baby milk.

Differences can be seen between the thermograms generated by the baby milk and Case 136, however there are a wide variety of baby milk brands and formulations. Also, Case 136 contained active drug substances and maybe other ingredients that would have interacted with the formulation, therefore creating differences in the trace.

Interestingly, Case 136 was the only case identified as containing chlordiazepoxide (**3**), making it different to the other illicit cases. GC-MS analysis also indicated that there may have been a small amount of diazepam (**1**) present in the tablets from this case but this was not confirmed by HPLC. It is likely that any diazepam (**1**) present

in the sample used for DSC analysis would have been obscured by the large endotherms produced by the lactose.

6.5.6 Baby Milk

Baby milk was further analysed in an attempt to determine if it would be possible to uncover the hidden interactions behind the large endotherms produced by the lactose. As the lactose produced such a large desorption curve at the beginning of the trace, which was likely to be hiding other thermal events, a sample was run using a hermetically sealed pan to prevent the water from leaving.

6.5.6.1 Methodology

Baby milk (10 mg) was analysed in a hermetically sealed pan using a thermal programme consisting of equilibration at 20 ^{o}C (1 minute) and then heat at 20 ^{o}C per minute to 220 ^{o}C.

6.5.6.2 Results of DSC Analysis

The sample of baby milk produced a complicated thermogram to 145 ^{o}C, followed by a sudden dip (Figure 6.12). A large exothermic peak was then produced before the sample degraded. The complicated line at the beginning of the trace is likely to be a result of the fats, oils and proteins within the milk formula, as well as their interactions, which will include melts, crystallisations and changes in the conformation of proteins. The dip at 145 ^{o}C is likely to be caused by the dehydration of the lactose monohydrate. Lactose is listed as one of the main constituents on the label of the baby milk tin (Cow & Gate, 2015).

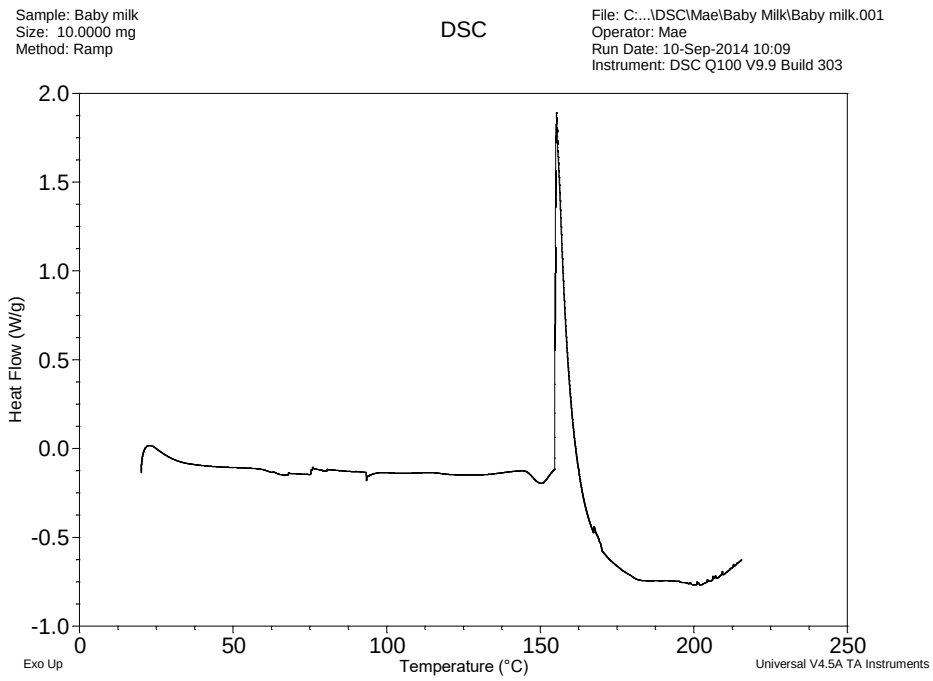


Figure 6.12. Thermogram produced by heating baby milk in a hermetically sealed pan.

6.5.7 Effects of Ageing

DSC analysis of the illicit cases continued over several months and it was noted that changes had occurred in the tablets. Analysis was performed on both powdered remains of the same tablets originally analysed, which had been stored in sealed vials, as well as analysis of fresh tablets taken from the same case. Very few of the cases that had originally shown amorphous lactose content, demonstrated the exothermic peak indicating the transition to crystallinity in the later samples. In addition, the endotherm produced by the dehydration of the monohydrate appeared slightly larger. It may be that over time the meta-stable tablets had become more crystalline and settled into this more stable form. The changes are demonstrated by thermograms from Case 79 in Figure 6.13.

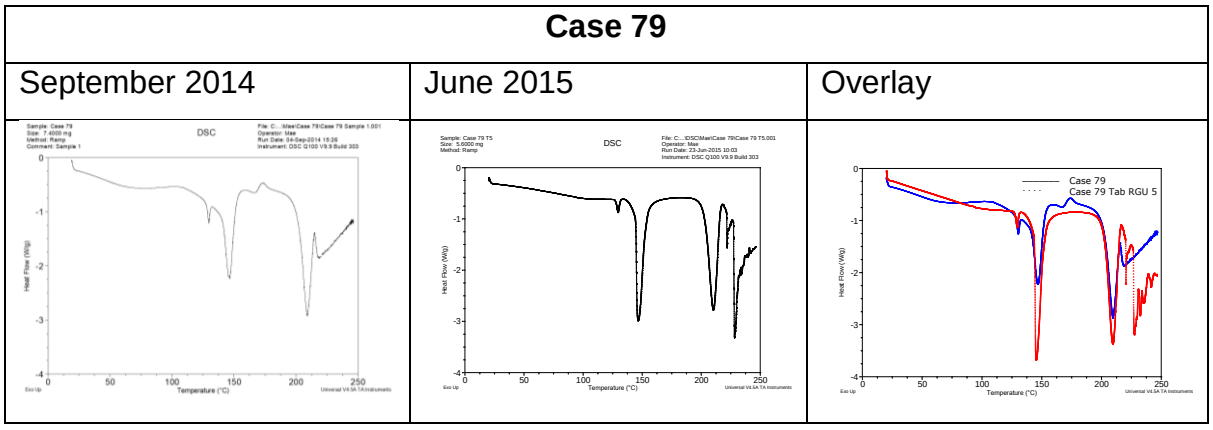


Figure 6.13. DSC thermograms produced by tablets from the same case generated 9 months apart showing the disappearance of the inflected transition relating to crystallisation, centred around 175 °C.

The difference in crystallinity was also demonstrated in the pharmaceutical tablets (Figure 6.14). The initial run of the tablets from this batch took place only one month before the expiry date given on the packet. Therefore, these tablets were already beginning to age, which may explain the slightly smaller crystal transition in the original trace. However, within sixteen months this exothermic event was no longer visible.

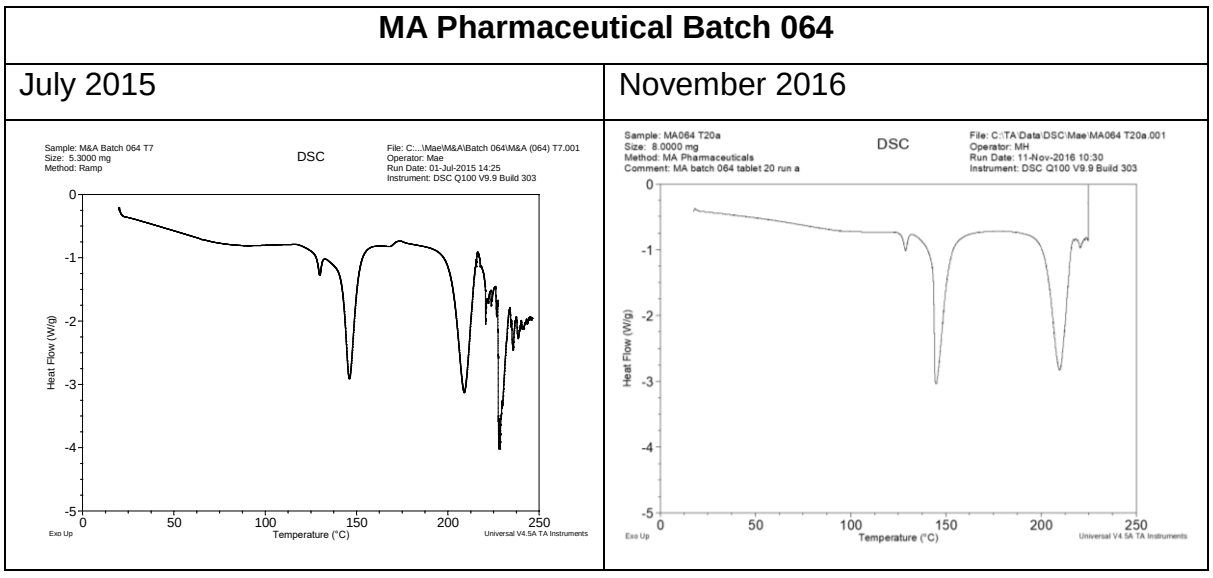


Figure 6.14. DSC profiles produced by tablets taken from the same batch of pharmaceutical tablets generated 16 months apart.

DSC analysis was also performed on the baby milk to identify any changes that might have taken place over time (Figure 6.15). Interestingly, this trace has begun to show similarities to the thermograms produced by the Group 7 cases, (84, 85 and 131) (Figure 6.10). There are still noticeable differences however but further work into the ageing of baby milk and maybe analysis of other brands could prove interesting in the future to gain insight into the behaviour of this material compared to lactose which is a major excipient in pharmaceutical and illicit tablets.

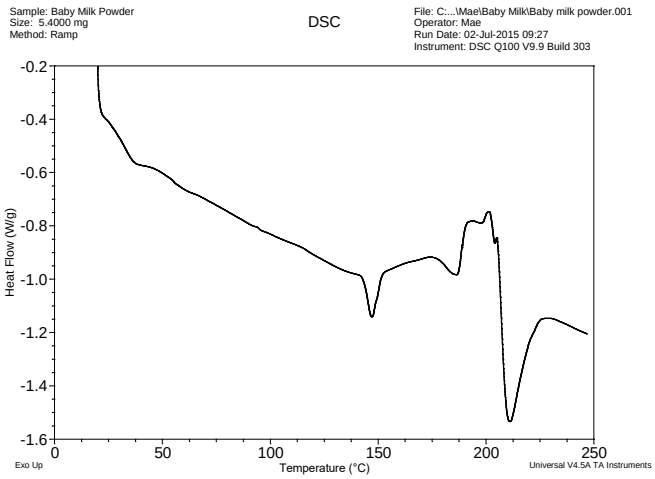


Figure 6.15. Thermogram produced by the same tub of baby milk as analysed in Figure 6.8 but analysed 10 months later.

6.5.8 Analysis of Results

The results of DSC analysis of the pharmaceutical and illicit tablets indicated that further separation could be made between the batches. By combining the data gained through previous investigations into the physical characteristics and chemical analysis using GC-MS and HPLC, with the groupings identified through comparison of the thermal profiles, a more complicated scenario of potential links emerged. This is demonstrated by the inclusion of DSC results to the Venn diagram (Figure 6.16).

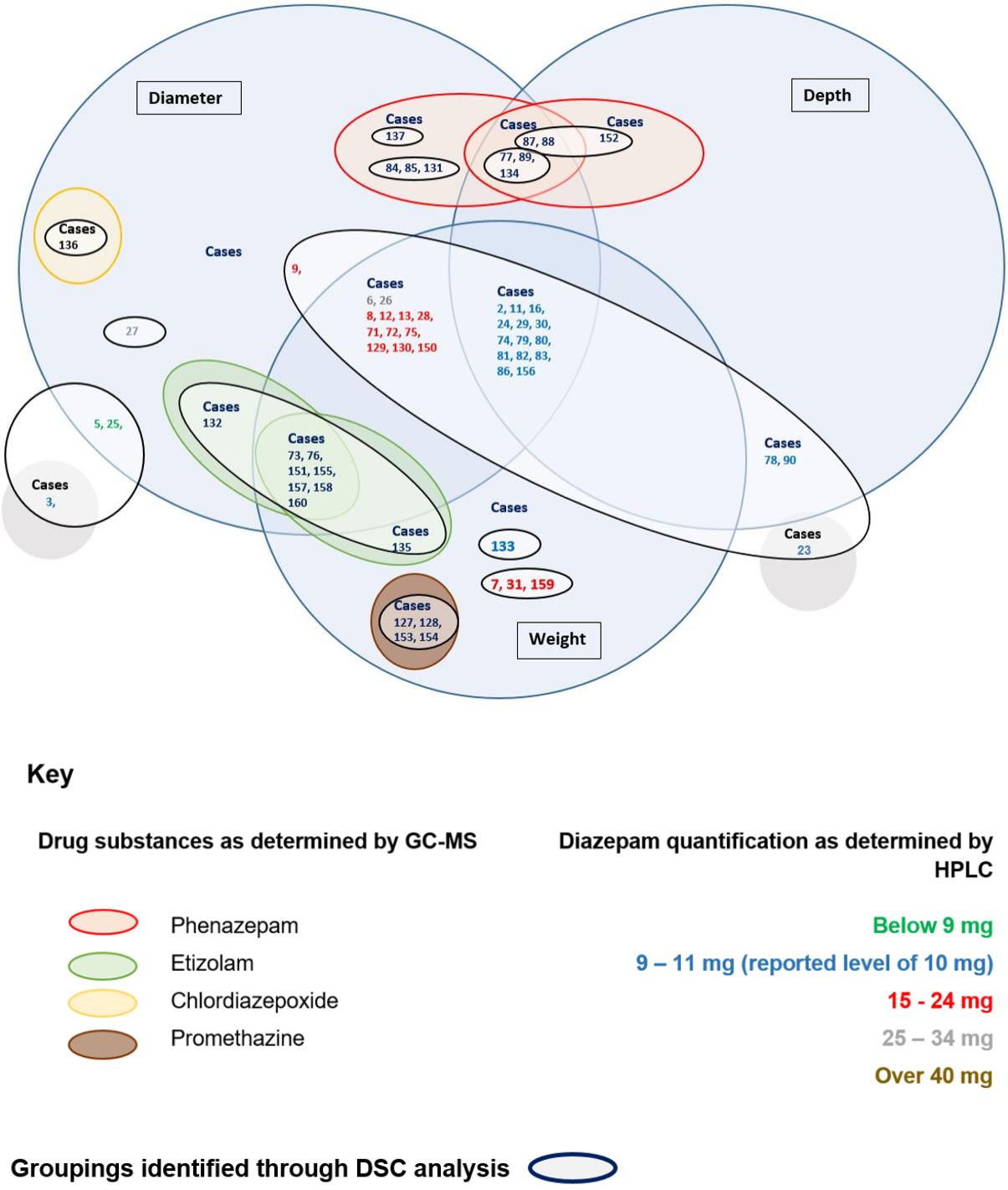


Figure 6.16. Venn diagram produced by combining the results of DSC analysis with the previous data obtained from physical analysis and the chemical techniques of GC-MS and HPLC.

Some of the groupings previously found to contain diazepam (**1**) by GC-MS analysis, such as cases 7, 31 and 159, are supported by DSC thermograms. This would indicate that no significant differences were detected in the excipients. However,

although DSC grouped these cases with the pharmaceutical tablets, they have been identified as illicit due to a higher level of diazepam (**1**) (15 – 24 mg). Differences in the level of diazepam (**1**) were not easily visible on the thermogram as the profile was largely dominated by lactose, indicating that these illicit tablets use a formula with some similarities to pharmaceutical tablets.

Other groupings of cases confirm the results of previous tests, such as cases 127, 128, 153 and 154, which were separated out from the remaining cases by all other techniques. Cases 3 and 23, however, produced distinct thermograms indicating differences between these two cases of tablets. Case 3 notably showed similarities to cases 5 and 25 which recorded a diameter similar to the pharmaceutical tablets but did not match with the pharmaceutical range in either depth or weight. Cases 5 and 25 also contained a level of diazepam (**1**) slightly lower than the pharmaceutical dose at 8 mg. It may be that if there is a link between cases 3, 5 and 25, that these are illicit tablets with an inconsistent blend of constituents or that they may have been pharmaceutical tablets that did not meet the accepted standard and were rejected. However, difference in measurements, lower diazepam (**1**) levels and a thermal profile distinct from the pharmaceutical tablets would suggest that these three cases were illicitly manufactured.

Case 23 produced a thermogram consistent with pharmaceutical tablets, along with cases 78 and 90. These three cases were found to contain a slightly lower level of diazepam (**1**) by HPLC analysis, which was rounded up to 9 mg to allow for errors of measurement. This meant that the diazepam (**1**) level was at the lower end of the pharmaceutical range. A difference in the physical measurements of the tablets had previously separated these cases from the known pharmaceutical batches, with cases 78 and 90 only showing consistency with the legitimate tablets in depth. Case 23 differed in all measurements.

Interestingly the cases which GC-MS analysis identified as containing phenazepam (**16**) have been separated into smaller clusters according to DSC analysis, with distinct differences being highlighted in the thermograms. This could indicate that the phenazepam (**16**) tablets were produced by multiple manufacturers or at different times, with a varying formula. However, this may require further investigation. As

the constituents of the phenazepam (**16**) tablets are unknown, it is not understood how differences in storage conditions over a period of time may affect the thermal profile of the illicit tablets. The initial investigations in this project have detected changes in lactose over a short period of time. It may therefore be the case that some of the phenazepam (**16**) tablets are linked but were manufactured at varying times or have experienced different storage conditions. Alternatively, it may be that there were differences in blend either during manufacture, or due to the small amount of sample analysed, the portion tested may not have been fully representative.

In contrast, the cases containing etizolam (**7**), two of which (cases 132 and 135) had been separated by measurements of diameter and weight, were grouped together by thermal analysis. The similarity in thermograms would indicate that there was no detectable difference in their excipients by DSC analysis. As filling of die wells and blend are more likely to be effected by human judgement and a difference in diameter of 0.01mm may be due to errors of measurement, it may be that there is a potential link between cases 73, 76, 132, 135, 151, 155, 157, 158 and 160.

Interestingly the DSC thermogram separated Case 133 into group 9. This case was noted during the investigation into physical characteristics as bearing markings consistent with tablets manufactured by the UK licensed pharmaceutical company Actavis. The tablets in this case had slight discrepancies in both weight and diameter with the known Actavis pharmaceutical tablets and also demonstrated a greater variation in measurements within the batch. However, the thermogram generated by DSC was consistent with those produced by the known pharmaceutical samples. As mentioned in section '6.5.3 – Pharmaceutical Tablets', the thermogram produced by the Actavis pharmaceutical tablets was distinct from all other known pharmaceutical samples tested in this project. This would therefore support the theory that Case 133 may contain pharmaceutically manufactured tablets that have been diverted into the illicit market. The thermogram produced by Case 133 also revealed only a small indication of the melt of diazepam (**1**) at 130 ^{o}C, which was concealed by the ***α***-lactose monohydrate dehydration. This supports the findings of the HPLC analysis which revealed that the tablets analysed from Case 133 had a lower level of diazepam (**1**), which was rounded up to 9 mg, to allow for errors of

measurement. This adjustment permitted Case 133 to reach the lower level of diazepam (**1**) accepted within the pharmaceutical range. The reason for the lower level of diazepam (**1**) being present may be due to degradation through age and / or storage conditions or the tablets may have originated from a rejected batch.

The group of tablets consistent with the known pharmaceutical tablets in the previous physical and chemical analysis performed in this project (cases 2, 11, 16, 24, 29, 30, 74, 79, 80, 81, 82, 83, 86 and 156), also produced DSC thermograms similar to the known pharmaceutical tablets. This continued to support the theory that these cases may be of pharmaceutical origin but have been diverted into the illegal supply chain.

6.6 Conclusion

DSC analysis into illicit tablets is an innovative use of an established technique and no previous work on this type of analysis has previously been published. It has proved to be an interesting and informative technique. It is a quick method of analysis requiring little preparation and unlike many of the other analytical methods used, which focus on the active drug substance, DSC provides a valuable insight into the excipients present within the tablets. This is particularly important when there is only a small amount of drug substance present.

Experiments have shown consistent results when multiple runs have been performed on the same tablet and for different tablets within a case. The technique was found to be sensitive enough to identify different concentrations of diazepam (**1**) within tablets, as can be seen on thermograms produced by two different Roche tablets. DSC could identify the tablets that contained diazepam (**1**) and distinguish between those which contained other active substances, alongside those which contain lactose as opposed to other diluents such as *Emcompress*™. Thus, DSC has the potential to show clear differences between the seized cases as well as showing similarities.

One of the major benefits of the technique however, is that the thermograms are largely influenced by the main excipients present within the tablets. It may be that illicit manufacturers include a variety of active drug substances as they become available but if they have a formulation of excipients that work, it is possible that the other constituents will remain the same. This could therefore produce a chemical profile which may help to identify potential links between illicit cases. This analysis has therefore proved valuable for distinguishing between batches of seized tablets in this project but has also provided an important insight into a technique that deserves further investigation for future studies.

In addition, although this chapter concentrated on visual comparison of DSC thermograms, the technique generated a vast dataset, allowing further analysis using k-means clustering and Principal Component Analysis (PCA). The statistical investigation compared finer detail missed by visual comparison. Both statistical techniques were performed for this project and discussed in Chapters 7 and 8 – 'Statistical Cluster Analysis' and 'Statistical Differentiation of Pharmaceutically Manufactured Tablets'. This project therefore, presents the benefits of using DSC for both visual and statistical analysis, making it a novel and innovative technique with great potential for future use in forensic studies.

Chapter 7. Statistical Cluster Analysis

7.1 Chapter Summary

The information gained from the physical and chemical tests was used to provide data for statistical and chemometric analysis. Agglomerative hierarchical clustering and k-means clustering were used to explore patterns in the data and identify potential groupings. The results of all of the experiments were then added to a heat map under the premise that the more individual cases cluster with other individual cases, as an indication of the probability of there being a potential link between them

7.2 Introduction

Analytical scientific techniques provide vital information regarding the chemical content of tablets but simple comparison of drug and excipient content cannot necessarily provide information regarding the origin of illicit cases. During this study, chemical and physical examination of illicit and pharmaceutical diazepam (**1**) tablets produced a rich dataset of measurements for each case on a range of variables. Within this dataset there are likely to be cases that are related because they were produced by the same manufacturer, illicit or otherwise. Cluster analysis uses the values in a multivariate dataset such as the one produced in this study to group similar observations (cases) based solely on their numerical values on a set of variables.

Statistical techniques involve both supervised and unsupervised methods. Supervised methods are used to distinguish between known numbers of groupings within the data when examples of each can be provided to build a training set, against which future samples can be tested. Examples of a supervised procedure include linear discriminant analysis, which is used in the following chapter to distinguish between known pharmaceutical tablets and those of unknown origin.

Unsupervised techniques are used to investigate patterns within the data without prior knowledge of potential groups, allowing clusters to be determined according to similarities between the samples alone (Clarke, 2009). In this chapter, cluster analysis will be used to reveal groupings within the illicit cases that may suggest

relationships between the groups. These groups will be decided objectively using only the numerical data produced by the analysis of the tablets' physical and chemical characteristics.

The clustering techniques used will be agglomerative hierarchical clustering (AHC) and k-means clustering. These techniques are well documented for identifying batches of illicit drugs, for example hierarchical cluster analysis was used by Ortiz (2012) for identifying clusters between illicit viagra and cialis tablets based on physical measurements of weight, thickness and length. In the study, the clustering technique clearly separated the cialis tablets into two groups, illicitly and pharmaceutically manufactured but, there proved less separation between the viagra tablets, where the authentic tablets were grouped with batches of illicit tablets. Principal component analysis and k-means clustering were used by Myors (1998) for 'heroin fingerprinting' and hierarchical clustering was also applied using 'manhattan' distances instead of euclidean. However, the findings of this study indicated that the results were comparable and supported each other.

In terms of this project, 'pharmaceutically manufactured' refers to tablets that have been legitimately manufactured for the UK market. Tablets originating from other sources may have entered the illicit market through the internet, making purchases from unknown suppliers across the world a viable option. Therefore, tablets that did not bear the markings of a legitimate manufacturer, known to have produced tablets for UK consumption, or tablets failing to meet the specifications regarding type and quantity of active drug substance, were deemed as illicit for this project.

7.3 Aim of Statistical Cluster Analysis

The aim of this study was to find related groups of illicit drugs and potential links between illicit and pharmaceutical tablets produced for the UK market by applying cluster analysis to the dataset of physical and chemical properties. Two objective clustering techniques were used, AHC and k-means and a subjective visual clustering of the tablets' appearance. A consensus was reached on the number of appropriate clusters.

Each clustering technique was carried out twice; one on the physical and chemical data and once on DSC thermogram data and combined with the subjective clustering produced five grouping outcomes. Each clustering attempt was carried out independently of the others and the results visualised in a heat map.

7.4 Procedure

7.4.1 Variables Used in the Analysis

The method of clustering is an exploratory data analysis method for grouping similar observations in multivariate data sets. In this case, the observations are the cases and there were three independent data sets used:

1. Subjective - colour, markings, general appearance and texture. Chemical analysis performed by high performance liquid chromatography was used to inform on type and level of active drug substance.
2. Physical – tablet diameter, thickness, weight and standard deviation of weight was also combined with information generated through chemical analysis regarding the type of active drug substance and amount of diazepam (**1**).
3. Differential Scanning Calorimetry data – 7211 heat flow measurements that form a thermogram, supported by the visual appearance of the thermograms provided an independent dataset that was heavily influenced by excipient content.

7.4.1.1 Subjective Comparison

Subjective separation was carried out in order to discriminate between cases according to physical appearance. Initially the seized cases were separated according to tablet markings, with thirteen different sets of embossed markings revealed. The largest group consisted of twenty-four out of the total sixty-five cases and had the overlaid logo MA on one side with D/10 on the reverse (Figure 7.1). MA and D/10 are the markings used by MA Pharmachem, a known pharmaceutical manufacturer that has supplied 10 mg tablets for the United Kingdom. Although these tablets are no longer produced by MA Pharmachem, the tablets may have been legitimately available at the time of seizure, due to the length of their shelf-life.

MA Pharmachem provided specification details of their diazepam (**1**) tablets along with two batches of pharmaceutically manufactured tablets for comparative purposes in this study. Therefore, these tablets became the focus of the statistical analysis as a valuable test sample group.

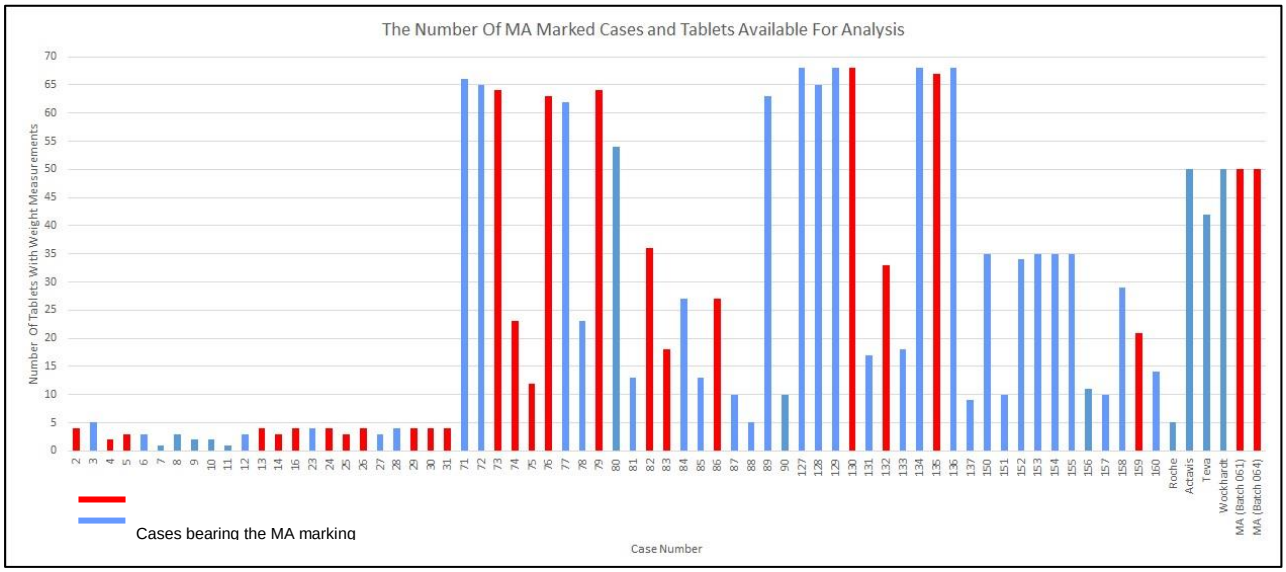


Figure 7.1. Bar chart showing the number of cases marked MA and D/10 and the amount of tablets within the cases. Cases with the MA and D/10 marking are shown in red and those with alternative marking are shown in blue. The named cases on the right of the chart identify pharmaceutically manufactured batches analysed for comparative purposes.

The illicit cases bearing the MA and D/10 mark were separated visually according to general appearance. Initially the style and size of font were considered but this was soon disregarded after correspondence with MA Pharmachem indicated that the company had previously produced tablets with some variability in the character size and appearance. Thus, this feature could legitimately vary between the known pharmaceutical tablets. Albeit, this allowance could be made, it was deemed unnecessary as the font remained constant within the groups that were identified through the visual clustering process. Two of the cases (case numbers 5 and 25) had a chalky appearance and others were a deeper blue (case 73) or more green in colour (case 5). The differences can be seen in Figure 7.2.

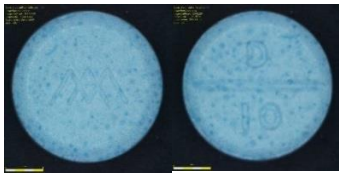
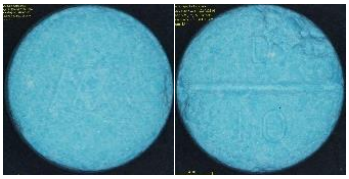
Case 2	Case 5	Case 159
		
Case 4	Case 73	MA Pharmaceutical Tablet
		

Figure 7.2. Photographs of tablets from different cases taken with the Olympus DSX100 opto-digital microscope. The MA pharmaceutical tablet was photographed using a Nikon D5100 camera with 36mm extension and 5000K lightbox.

Data regarding the identification of active drug substance by gas chromatography-mass spectroscopy (Chapter 4) and quantification of diazepam (**1**) using high performance liquid chromatography (Chapter 5) was analysed in combination with the visual differences to gain extra insight. This allowed tablets containing the expected 10 mg of diazepam (**1**) to be grouped together and those with a higher or lower concentration to be placed with one another. Likewise, the tablets containing phenazepam (**16**) and those with etizolam (**7**) were grouped accordingly (Figure 7.3).

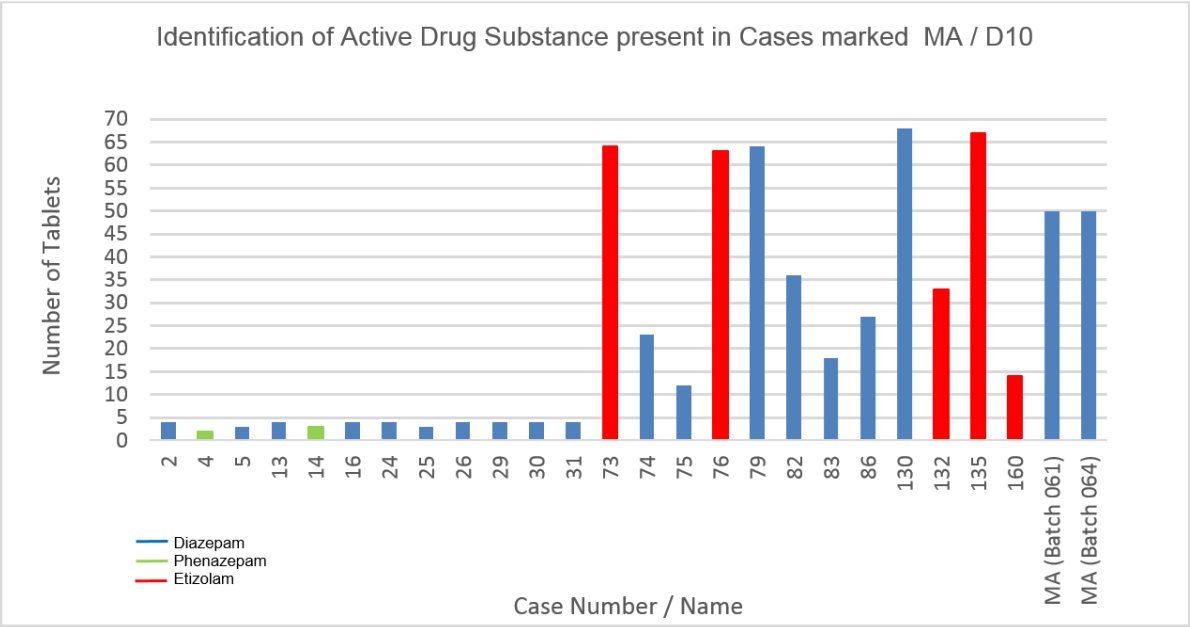


Figure 7.3. Bar chart showing the number of available tablets within each case marked MA and D10, including the two batches of pharmaceutically manufactured tablets, labelled at the end. The blue coloured lines indicate the cases that contained diazepam (1), the green identifies those that contained phenazepam (16) and the red represents those with etizolam (7).

7.4.1.2 The Physical Characteristics

The physical characteristics of the tablets (weight, diameter and thickness) were measured three times and the mean of each tablet was added to the database. Although, the diameter of the tablets was recorded, there was found to be very little variation between the illicit tablets and their known pharmaceutical counterparts. Therefore, this was not used in the statistical analysis. However, data relating to tablet thickness and weight was extracted from the database and analysed in conjunction with the chemical information regarding drug content and quantification.

Tablet weight was used in two ways. Firstly, by comparing the mean weight of the tablets in each case and secondly by calculating the relative standard deviation of weight from the tablets in each case. It is acknowledged that only a small sample size of four tablets from each case was used for the weight analysis and was found to be a limitation of this project. Case sizes collected by the police are variable and the small sample size reflects the fact that many of the cases contained fewer than

five tablets (Figure 7.3). Therefore, four tablets were selected from each case in order to maximise the number of cases that were available for analysis.

It was noted that having such a small sample size for weight was a limitation with this project. In order to test the viability of using only 4 tablets from a case, the analysis was performed with five different sets of four tablets from each case, when the quantities in the case so permitted. However, this did mean that the cases which contained only four tablets continued to use data from the same four tablets. If the four randomly chosen tablets were a representative sample, then the clustering should essentially remain consistent.

7.4.1.3 Differential Scanning Calorimeter

Pharmaceutical diazepam (**1**) tablets contain only 10 mg of active drug substance, with excipients making up the bulk of the tablet. The Differential Scanning Calorimeter (DSC) measured changes in heat flow against temperature as a sample was heated, creating a thermogram based on the data points. Endothermic and exothermic peaks were created according to thermal events taking place in the heated sample and varied in location and height according to the substances and quantities present (Figure 7.4). DSC analysis therefore demonstrated the capacity to identify differences between tablets based largely on their excipient content (Chapter 6 - Differential Scanning Calorimetry). The data points generated by DSC were converted to an excel spreadsheet and analysed as an independent dataset. Although DSC thermograms ranged from 22 – 240 ^{o}C, only the 7211 data points relating to the temperatures between 100 ^{o}C – 220 ^{o}C were used. This was because the lower temperature was affected by evaporation of water and solvent content from the samples and degradation manifested at higher temperatures did not characterise specific components within the tablets (Bibi *et al.,* 2015).

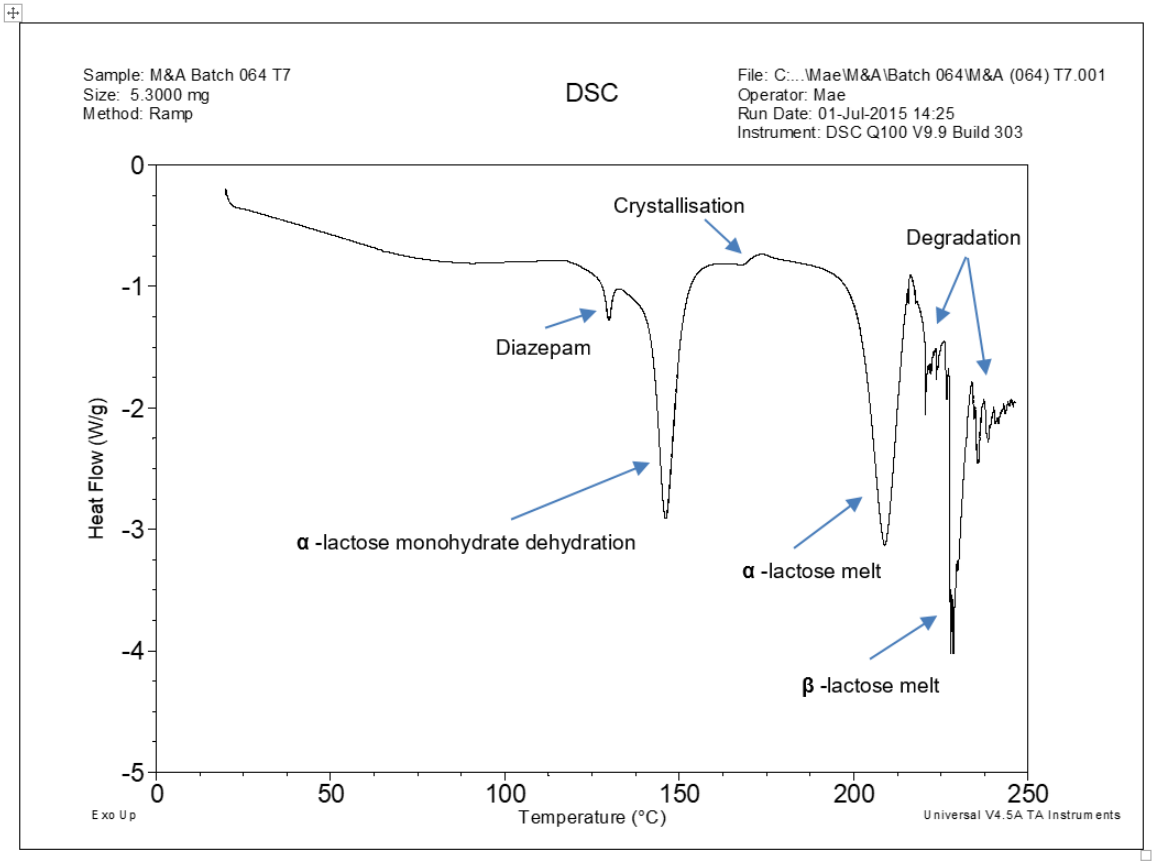


Figure 7.4. DSC Thermogram of a 10 mg pharmaceutical tablet produced by MA Pharmachem. The main peaks are identified but vary in size and location according to the components present.

7.4.2 The Clustering Techniques

A variety of statistical techniques were applied to the data produced by the physical and chemical analyses. The intention was to demonstrate that the more frequently two cases clustered together, the greater the likelihood of there being a potential link between the two cases. The clustering techniques used were agglomerative hierarchical clustering (AHC) and k-means clustering. AHC utilised the principle of single linkage clustering to identify the different groupings, whereby each sample is clustered with its nearest neighbour according to euclidean distance. K-means clustering also uses euclidean distance but groupings were determined by comparing each object to the cluster mean. Worked examples of both AHC and k-means clustering are demonstrated in the Appendix III. The results of these

techniques were combined on a heat map to visualise results and determine whether repeats of the same groupings could identify potential links between the cases.

7.4.2.1 The use of Subjective Clustering

The physical appearance of the tablets was examined and a subjective assessment was made regarding colour and texture of the tablets. Although description of colour is very subjective, noticeable differences between the shades of blue was very apparent, allowing tablets to be separated. Variations in texture was also visible, with cases 5 and 25 appearing more chalky. Again, this was subjective because the cause of the powdery nature of the tablets within the cases was unknown and may have been due to constituents within the tablet or storage conditions.

Differences in font were not used for separation because it was acknowledged that pharmaceutical companies may use a variety of fonts. Interestingly, the cases did separate according to font when chemical information was added. The identification of active drug substance (by GC-MS) and quantification of diazepam (**1**) (by HPLC) allowed further separation to made between the seized cases and all of this information combined into five distinct groups.

7.4.2.2 The use of Agglomerative Hierarchical Clustering (AHC)

AHC uses a single-linkage technique to classify continuous data, whereby each observation (case) starts off as being separated from the rest. For each step, Euclidean distance is used to locate its nearest neighbour(s) and the observations are grouped together. The process continues until all observations are grouped into one large cluster (Kogan, 2006). The number of groups is determined by common sense relating to background information relating to the data explored. For this study, the number of clusters was informed by the subjective clustering, in order to provide comparable data for the heat map. However, the dendrograms were studied for additional intelligence generated by the data.

The data was standardized using SPSS to create a Z-score. This was done to avoid the data being weighted towards the higher numbers and allow each variable to contribute equally to the result. Standardization was performed by subtracting the

mean from each variable and then dividing the remainder by the standard deviation, according to the equation:

$$Zx = \frac{Xi - X}{Sx}$$

(Geert van den Burg, R., 2017).

AHC was performed using SPSS and a range of solutions from 2 - 10 clusters was requested, in order to explore any alternative groupings present. The method of nearest neighbour was used and the results were visualised in form of a dendrogram.

7.4.2.3 The use of K-means Clustering

K-means is a clustering technique used to analyse continuous data through Euclidean distance in a similar way to AHC. However, k-means uses a cluster centroid to locate the nearest neighbour. As observations (or cases) are added to the cluster, the centroid is recalculated for the following iteration (Kogan, 2006).

The number of clusters has to be stipulated by the user based on knowledge of the data and background information and no alternatives are provided by the analysis. This therefore has the potential of being affected by outliers. Outliers may be forced into clusters with nearest neighbours that may be some distance away, instead of forming a new cluster. As the centroid is then recalculated, the nature of the cluster is altered and distorted (Liu and Lu, 2015). This is demonstrated in Figure 7.5.

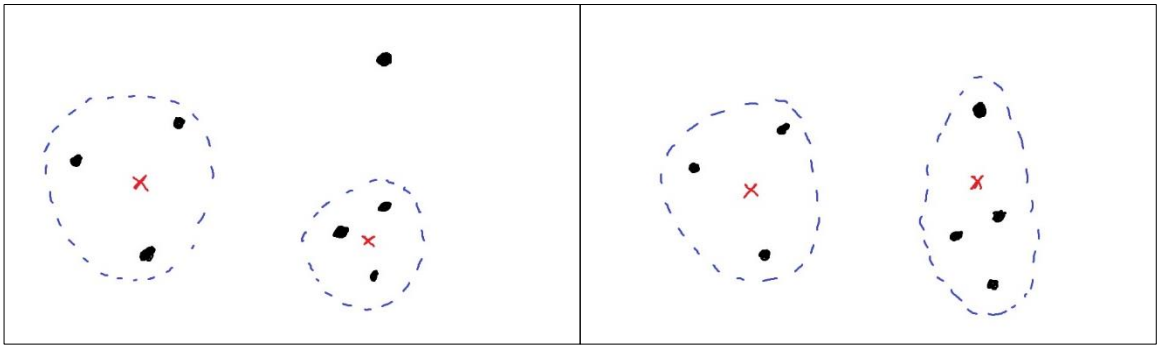


Figure 7.5. The effect of outliers on k-means clustering. In the first diagram two clusters can be identified, leaving the outlier. The cluster centre is marked in red. In the second diagram, the outlier has been incorporated into the second cluster, thus moving the centroid.

The problem of distortion is less of an issue for AHC. Using AHC, as the clusters increase in size new members are connected to the nearest neighbour, not to a centroid and cluster distance can be visualised on a dendrogram. Therefore, in order to help alleviate the problem caused by outliers, the number of clusters requested for the k-means was based on the results of the AHC dendrograms. K-means clustering was performed using SPSS with 20 iterations using running means.

7.4.3 Heat Map Creation

The results of each of the clustering techniques were combined in a heat map. This allowed a comparison of results to be performed with the aim of highlighting cases that consistently grouped together, given the three independent sets of variables and three different clustering techniques applied.

Two cases consistently clustering together suggests that the two have similar features and, in this study, given that the features relate to physical and chemical parameters, this implies the tablets have similar chemical and physical features. It is therefore possible that the two cases may be related i.e. they may be produced by the same manufacturer or maybe from the same batch.

7.5 Results and Discussion

7.5.1 Results of Subjective Clustering

Examination of the tablet appearance in conjunction with chemical data regarding active drug substance, allowed the seized cases to be separated into five groups. Ten cases clustered in Group 1. Group 1 cases contained tablets similar in appearance to the batches of pharmaceutical tablets provided by MA Pharmachem and contained the anticipated 10 mg of diazepam (**1**).

The six cases placed in Group 2 contained more than double the expected amount of diazepam (**1**) with over 20 mg and are almost certainly illicitly manufactured.

Group 3 contained the two tablets with a chalky appearance but also contained a slightly lower level of diazepam (**1**) (8 mg instead of the purported 10 mg). Both the drug substance level and the tablet appearance may be due to tablet content and manufacturing procedure or they may have been affected by deterioration and degradation over time.

Two cases were placed together in Group 4 and four cases in Group 5. The cases in Groups 4 and 5 did not contain diazepam (**1**) but contained different active drug substances, phenazepam (**16**) and etizolam (**7**) respectively, which are not licensed in the UK and so by definition originate from an illicit manufacturer. The results of the subjective groupings are listed in Table 7.1.

Table 7.1. The results of the subjective groupings of the tablets marked 'MA' and 'D/10'.

	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5
DIAZEPAM LEVEL (mg)	~10	>20	~8	0	0
OTHER CHARACTERISTICS			Chalky	Phenazepam	Etizolam
CASE NUMBERS	2	13	5	4	73
	16	26	25	14	76
	24	31			132
	29	75			135
	30	130			
	74	159			
	79				
	82				
	83				
	86				

7.5.2 Results of AHC

7.5.2.1 Active Drug Substance and Physical Data

Agglomerative Hierarchical Clustering (AHC) was used to define groupings in the illicit cases using the variables of the mean weight and relative standard deviation of weight from a random sample of four tablets taken from each case and the quantity of diazepam (**1**) measured by HPLC (Chapter 5). The two batches of known MA pharmaceutical tablets MA 061 and MA 064 were analysed alongside the illicit tablets for comparative purposes.

Three clusters were requested in the analysis based on the subjective clustering of the MA marked cases. Subjectively these tablets had been separated into five groups; however neither cases 4 and 14 nor cases 5 and 25 contained four tablets where weight measurements had been available. Therefore, these cases were not included in the AHC or k-means clustering of the physical data combined with the diazepam (**1**) quantification. As these four cases alone made up two of the groups identified by subjective clustering (Groups 3 and 4), it meant that three clusters would have been expected, with Group 1 visually grouping alongside the pharmaceutical tablets.

Both standardised and non-standardised data was tested (see section 7.3.2.2. – AHC) and the results indicated that when the data was not standardised, the diazepam (**1**) quantification dominated the clusters and the groupings reflected the concentration levels. However, when the data was standardised the majority of cases clustered together, with a few outliers. Although the outliers were shown as individual groups, clusters could still be identified to support the subjective clustering results. This was shown in the dendrograms produced by the AHC of the MA marked cases as demonstrated in Figures 7.6 and 7.7. In Figure 7.6 three clusters can be identified at a distance measure of 20, which is shown as a red dotted line. The three clusters represent cases that contained 10 mg of diazepam (**1**), those with no diazepam (**1**) but etizolam (**7**) as the active drug substance and cases that contained over 20 mg of diazepam (**1**). Figure 7.7 shows the same three clusters at a distance measure of 4 (shown as a red dotted line) but also identifies two outliers. The

number of groups were determined by the euclidean distance chosen. This is an exploratory approach and there is no definitive answer about determining the distance used. It is context dependent and is largely decided through logic. In this case, the clusters seen in Figures 7.6 and 7.7 at a distance of about 20 and 4 respectively are consistent and support the visual groupings; however, outliers appear dependent on the tablets chosen.

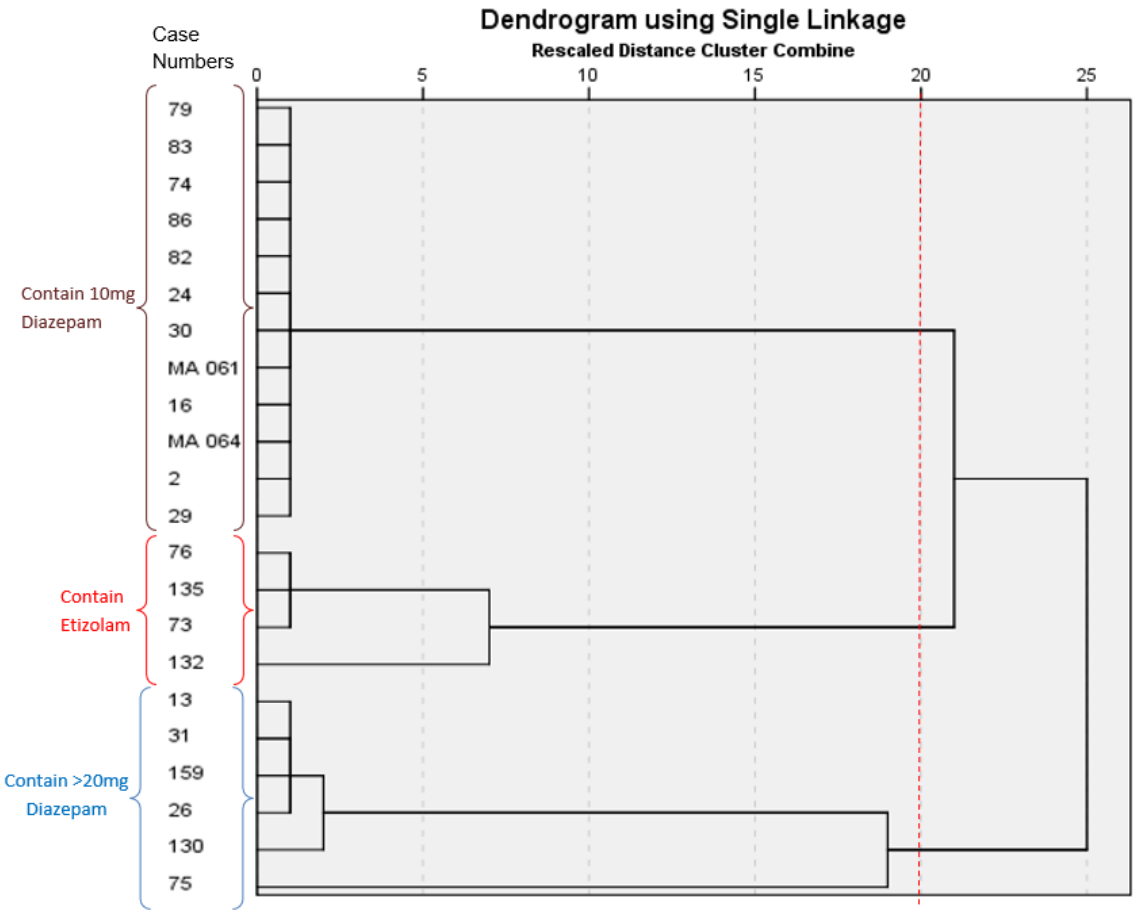


Figure 7.6. Dendrogram of MA marked tablets using non-standardised data. The 3 clusters stipulated (which can be seen at a distance of about 20, shown by a red dotted line) identify low or no diazepam (1) content, the expected 10 mg of diazepam (1) and the cases with a high concentration of diazepam (1).

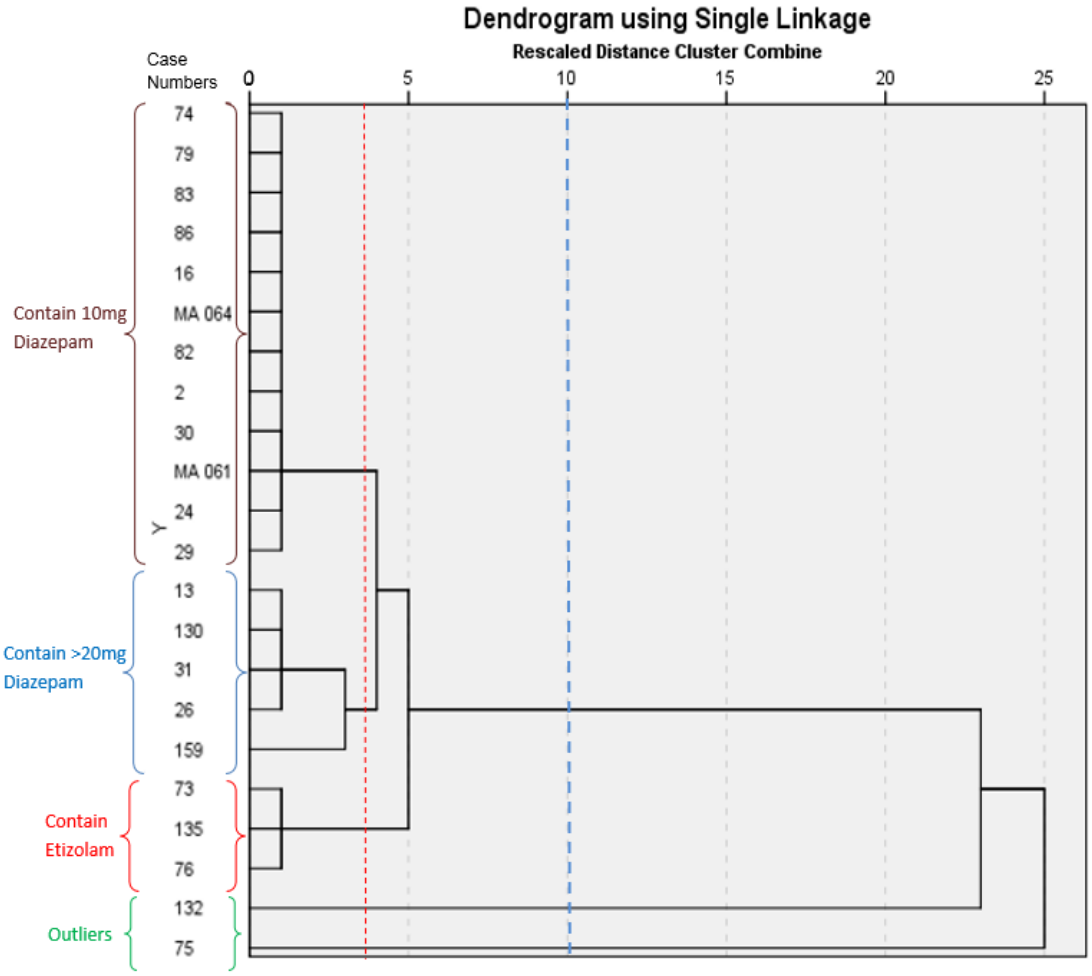


Figure 7.7. Dendrogram of MA marked tablets using standardised data. Although groupings containing the different concentrations of diazepam (1) can be seen, the three clusters stipulated identify one main cluster and two outliers and can be seen at a distance of 10 (blue dotted line). However, at a distance of 4 (red dotted line), the identified clusters support the visual groupings plus the two outliers.

7.5.2.2 Sample Size Test

The clustering was repeated using a different random sample of four tablets to evaluate whether such a small sample was representative for the case from which it was extracted. In total, the test was run five times, with a different sample of four tablets taken from each case, every time. The results were similar to previous dendrograms (Figures 7.6 and 7.7), which indicated that when a tablet was identified in Cluster 1 at a distance measure of about 10, it appeared to support the visual groupings, whereas clusters 2 and 3 appeared to be outliers. The cases identified as outliers changed each time, according to the four tablets chosen for the weight analysis. Each case fell within the same grouping each time, unless it was identified as an outlier (Figure 7.7). Table 7.2 shows how many times each of the cases clustered in a group other than the main Cluster 1. Eight of these separations occurred during the third run of the test. The group in which each case clustered is also identified.

Table 7.2. Cluster membership for each case using five different samples containing four tablets. According to the dendrogram shown in Figure 7.7, groups 2 and 3 appear to be outliers. The cases separated from the main cluster changed each time according to the four tablets chosen for the weight analysis, indicating some variation within each case.

	Cluster number					
Case Number	Run 1	Run 2	Run 3	Run 4	Run 5	Nº of times case separates from main cluster
2	1	1	1	1	1	0
13	1	1	1	1	1	0
16	1	1	1	1	1	0
24	1	1	1	1	1	0
26	1	1	3	1	1	1
29	1	1	3	1	1	1
30	1	1	1	1	1	0
31	1	1	1	1	1	0
73	1	1	2	1	1	1
74	1	1	1	1	1	0
75	1	1	1	1	3	1
76	1	1	2	3	1	2
79	1	3	1	1	1	1
82	2	1	3	1	1	2
83	1	1	1	1	1	0
86	3	1	1	1	1	1
130	1	1	1	1	1	0
132	1	2	2	2	2	4
135	1	1	2	1	1	1
159	1	1	3	1	1	1
MA 061	1	1	1	1	1	0
MA 064	1	1	1	1	1	0

In effect, the different cases which kept falling as outliers were largely inconsistent, with only 3 of the cases being identified as belonging to a different cluster on more than one occasion. Case 132 was the only case identified more than twice, with four separate samples being identified as inconsistent with the main group. These findings would indicate that there was some variability in weight between the tablets in a single case and therefore a sample set of four would not be ideal for producing the most consistent results in this type of analysis. However, as explained in section 7.3.1.2 (Physical Characteristics), due to the small number of tablets in some cases,

there was little choice but to use a small sample and a compromise was made between maximizing number of cases and maximizing sample size.

For comparative purposes the statistical analysis was also run using all of the available weight measurements for every tablet in each case, alongside the drug substance content. This was performed on all MA marked cases containing two or more tablet weights. As this method included cases 4, 14, 5 and 25, five clusters could be stipulated. The results are listed in Table 7.3. However, as the standard deviation of the tablet weight is affected by the number of cases included, this data was removed before analysis.

Table 7.3. AHC cluster membership for cases marked MA and D/10 using all tablets where a weight had been recorded. The cases marked MA061 and MA064 are tablets from two different pharmaceutical batches.

	Case Numbers				
Statistical Analysis	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
AHC Standardised	2,16,24,29,30,74,79, 82,83,86, MA061, MA064	4,5,73,76,132,135	13,26,31,75,130,159	14	25
AHC Non-Standardised	2,16,24,29,30,74,79, 82,83,86, MA061, MA064	4,5,14,73,76,132, 135	13,26,31,130,159	75	25

The results were produced using both standardised and non-standardised data and demonstrated some differentiation between cases. Standardised data generated results where clusters 1 and 3 were identical to those formed by subjective analysis. Clusters 4 and 5 identified Cases 14 and 25 as being different from other cases and cluster 2 contained the cases comprising of etizolam (**7**) as the active drug substance. However, it also misclassified cases 4 and 5 along with the etizolam (**7**)

cases. Potentially, if these cases were illicitly made, the same formula could have been used with different active drug substances, therefore giving a similar weight.

Case 5, which contained diazepam (**1**), had been visually grouped with Case 25 due to its chalky appearance and Case 4 had been previously clustered with Case 14 as they both contained phenazepam (**16**). However, the AHC cluster analysis categorised these cases as being different. The difference between the clusters are recorded in Figure 7.8.

The non-standardised data clustered cases 4 and 14, which contained phenazepam (**16**), in Group 2, with the cases containing etizolam (**7**) and separated case 75.

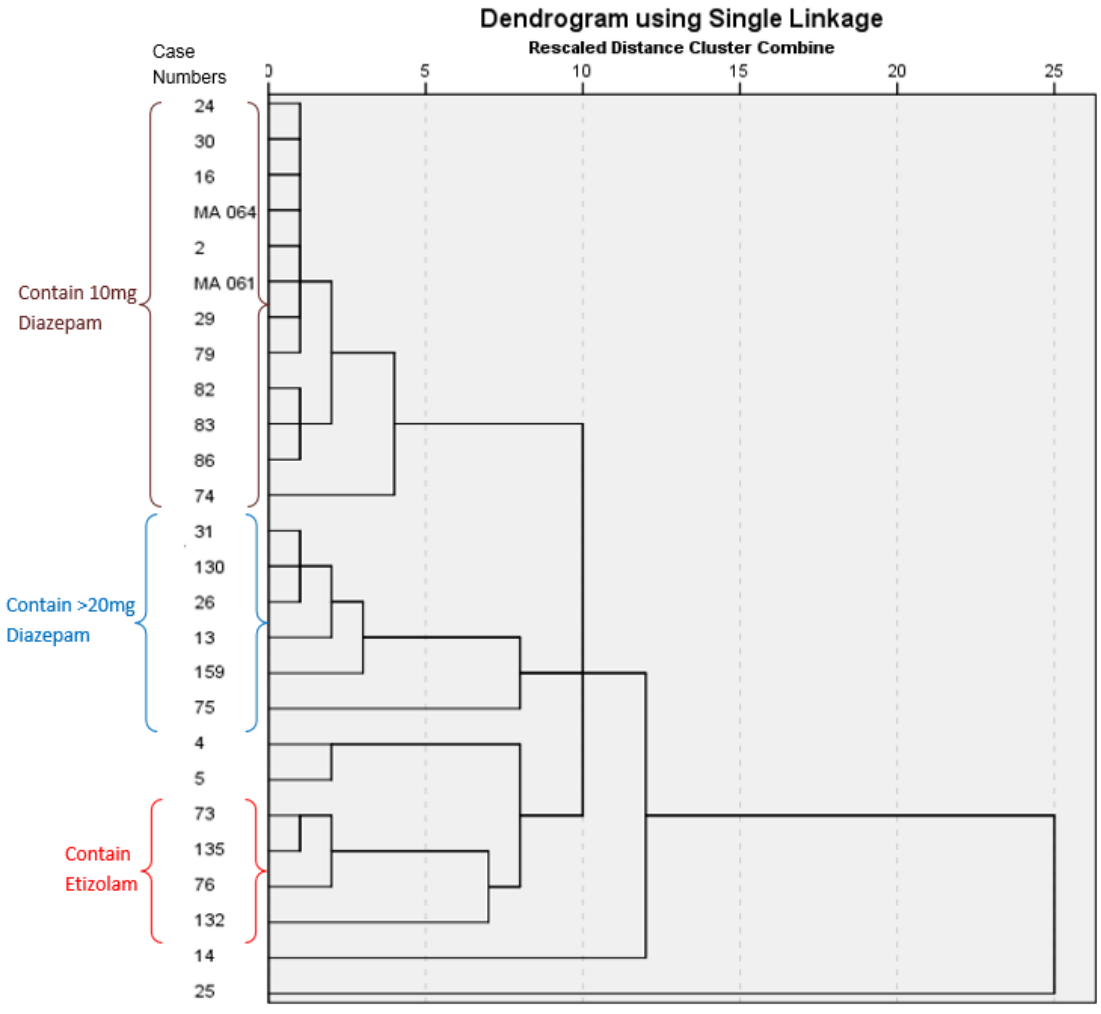


Figure 7.8. Dendrogram of all MA marked tablets using all available tablet weights and standardised data.

The dendrogram of all tablets does contain a variety of case sizes. The mean of all tablets within a case provides a centroid, with the larger cases forming a larger population in the cluster and a more accurate centroid on which to base the analysis. However, AHC is an unsupervised method of clustering which relies only on similarities within the dataset to form clusters rather than previous knowledge of the tablet origins, so there can be no certainty that the tablets within the suggested groupings bear any relationship with each other, except for their similar properties.

7.5.2.3 Analysis of Chemical Test Results Generated by DSC

DSC thermograms show the data generated by heat flow against temperature at a given time. The data related to temperatures lower than 100 ^{o}C and above 220 ^{o}C were removed from the dataset (see section 7.3.1.3 - Differential Scanning Calorimeter) and the remaining data was standardised and compared using both AHC and k-means clustering.

Initially the MA marked tablets were tested by AHC and the results indicated that for five clusters, all cases tended to group together except for four cases, which formed their own individual clusters as shown in the dendrogram in Figure 7.9. The five clusters can be seen at a distance measure of about 13 and is indicated with a red dotted line. The cases clustered individually were numbers 4, 14, 5 and 25, which subjective analysis had anticipated as being different but clustered in pairs. However, a closer look at the resulting dendrogram (Figure 7.9) proves interesting.

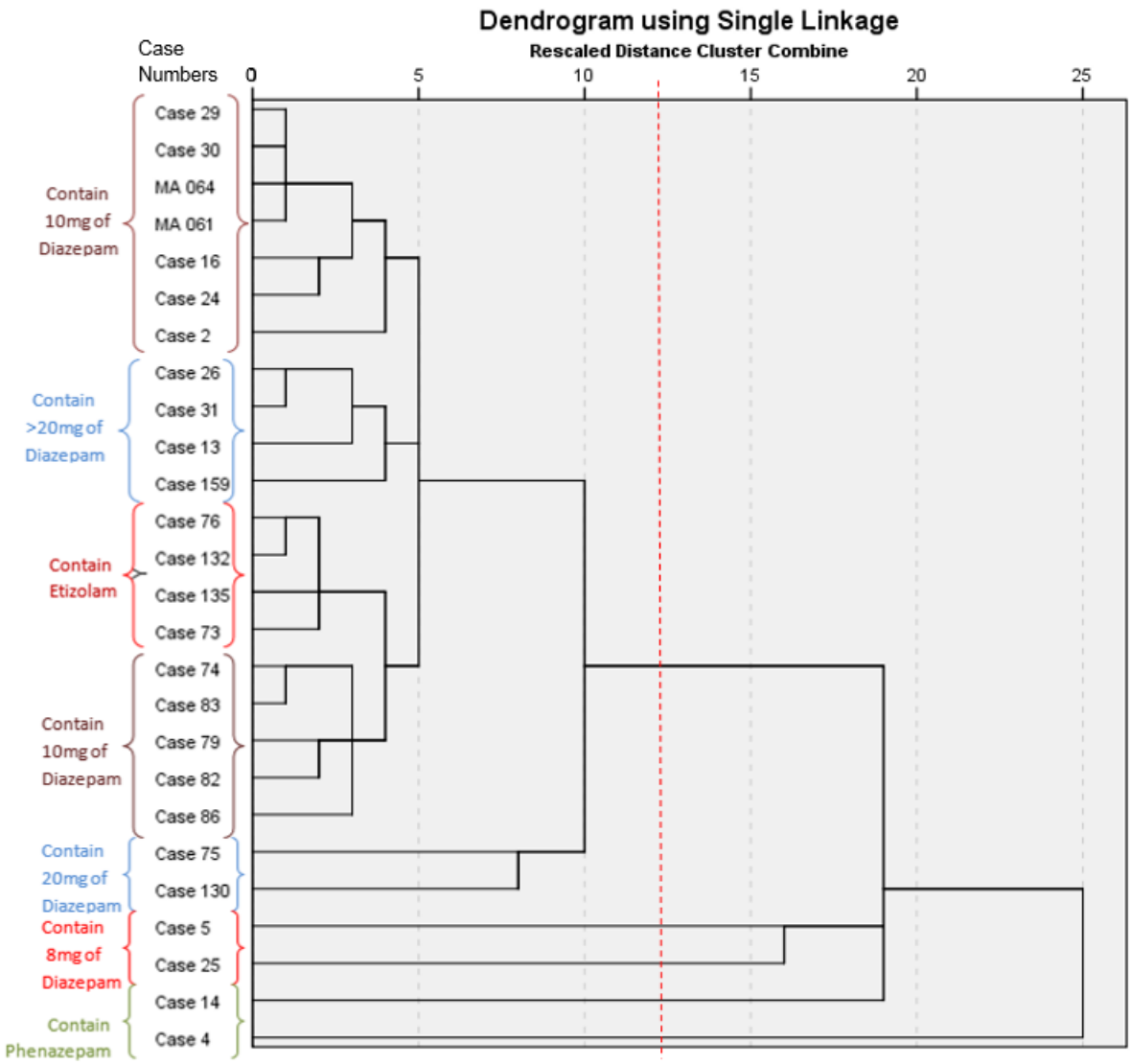


Figure 7.9. Dendrogram of the MA marked cases generated using data produced by DSC.

The dendrogram based on DSC data was generated from DSC thermogram data alone and contains no further input, such as drug quantification. Each thermogram is composed of 7211 sample heat flow values at a temperature between 100 and 220 ^{o}C. The thermogram is based on the reactions of the sample constituents, the majority of which are excipients rather than the active drug substance itself. Although the five clusters suggested by the subjective comparison are not entirely consistent with DSC results, the different clusters are still visible at the smaller distances with the subjective groups 1 and 2 being split into two clusters

each. Interestingly, the tablets in Cluster 1, which were considered to be potential pharmaceutical tablets in the subjective cluster have been separated. Due to potential errors of measurement and precision, during hplc analysis, all cases found to contain between 9-11 mg of diazepam (**1**) had a reported level of 10 mg for this project. However, in terms of DSC results, the cases which had been found to contain around 10 mg of diazepam (**1**) in the HPLC analysis, clustered very closely with the pharmaceutical tablets in DSC clustering. However, those with a recorded weight closer to 9 mg of diazepam (**1**) during HPLC analysis (cases 74,79,82,83 and 86), clustered separately based on DSC results. 9 mg is slightly low for the drug concentration permitted within the pharmaceutical industry but the level is close enough that it may be a result of degradation through ageing of the tablet, or maybe due to mathematical or analytical errors e.g. rounding of figures, or experimental inconsistencies, therefore they were reported as 10 mg tablets with the potential to be pharmaceutical. It is therefore interesting that DSC data identified a variation in these cases based largely on the excipient content, when no extra information regarding drug substance or quantification was included in this analysis. Cases 4, 14, 5 and 25 were notably different to the other cases.

The nine clusters (including outliers) at a distance of 5 were taken as the results of the AHC cluster analysis of DSC data, for the generation of the heat map in section 7.4.4.

7.5.3 Results of K-means Clustering

7.5.3.1 Active Drug Substance and Physical Data

K-means clustering of the physical data and chemical information regarding active drug substance was tested in both standardised and non-standardised form. This was based on the mean weight and standard deviation of weight, along with quantity of diazepam (**1**) from four randomly chosen tablets within each case. K-means produced the same results as the AHC analysis and indicated that non-standardised data was heavily influenced by diazepam (**1**) quantification, whereas standardised data produced one main cluster and isolated ungrouped cases.

7.5.3.2 Sample Size Test

The results of the sample size test, where a different four tablets were randomly chosen from each case when the required data was available, revealed that the cases which clustered apart from the main group were not necessarily identical to those separated by AHC. The results are shown in Table 7.4, which shows that fifteen more cases were separated from the main cluster using k-means clustering than with AHC. In each of these fifteen instances, the cases were separated away from Group 1 which contained the known pharmaceutical batches. Interestingly, with the exception of Case 29 in the fourth run of the experiment, all of the remaining separations were cases which had been subjectively differentiated from the pharmaceutical tablets based on the level of diazepam (**1**) present and the type of active drug substance. These cases were therefore likely to be illicitly manufactured and k-means detected a greater variability within them.

Table 7.4. Cluster membership for each case using five different samples of four tablets, indicated by AHC and k-means cluster analysis. The cases separated from the main cluster changed each time according to the four tablets chosen for the weight analysis, indicating some variation within each case. Cluster membership that differed between the groupings identified by AHC and k-means are highlighted in yellow. The case numbers at the side are coloured according to the subjective clustering technique, whereby the dark blue represents Group 1 which contain 10 mg of diazepam(1), pale blue represents Group 2 which contain over 20 mg of diazepam(1) and the pink represents Group 5 which contain etizolam (7).

	Cluster number AHC						Cluster number k-means					
Case Number	Run 1	Run 2	Run 3	Run 4	Run 5	Nº of times case separates from main cluster	Run 1	Run 2	Run 3	Run 4	Run 5	Nº of times case separates from main cluster
2	1	1	1	1	1	0	1	1	1	1	1	0
13	1	1	1	1	1	0	1	1	1	3	1	1
16	1	1	1	1	1	0	1	1	1	1	1	0
24	1	1	1	1	1	0	1	1	1	1	1	0
26	1	1	3	1	1	1	1	1	3	3	1	2
29	1	1	3	1	1	1	1	1	3	3	1	2
30	1	1	1	1	1	0	1	1	1	1	1	0
31	1	1	1	1	1	0	1	1	3	3	1	2
73	1	1	2	1	1	1	1	2	2	1	2	3
74	1	1	1	1	1	0	1	1	1	1	1	0
75	1	1	1	1	3	1	1	1	3	3	3	3
76	1	1	2	3	1	2	1	2	2	2	2	4
79	1	3	1	1	1	1	1	3	1	1	1	1
82	2	1	3	1	1	2	2	1	3	1	1	2
83	1	1	1	1	1	0	1	1	1	1	1	0
86	3	1	1	1	1	1	3	1	1	1	1	1
130	1	1	1	1	1	0	1	1	1	3	1	1
132	1	2	2	2	2	4	1	2	2	2	2	4
135	1	1	2	1	1	1	1	2	2	1	2	3
159	1	1	3	1	1	1	1	1	3	3	1	2
MA 061	1	1	1	1	1	0	1	1	1	1	1	0
MA 064	1	1	1	1	1	0	1	1	1	1	1	0

Cluster analysis of the weight measurements from every tablet in each case, along with data regarding the level of diazepam (**1**) present indicated that k-means clustering was not as discerning as the AHC. The k-means analysis using standardised data misclassified three cases containing etizolam (**7**) with the pharmaceutical tablets (Table 7.5). However, using the non-standardized data, the etizolam (**7**) cases are separated out, along with the phenazepam (**16**) tablets and the chalky Case 5. Interestingly, this technique differentiated between the pharmaceutical tablets and cases 74, 82, 83, 86 which all contained 10 mg of diazepam (**1**) and had been subjectively comparable.

Table 7.5. Cluster membership for cases marked MA and D/10 analysed by k-means and using all tablets where a weight had been recorded. The cases marked MA061 and MA064 are tablets from two different pharmaceutical batches.

	Case Numbers				
Statistical Analysis	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
K-means Standardised	2,16,24,29,30,73,76, 79,135, MA061, MA064	4,5,14,132	13,26,31,130,159	74,75,82,83,86	25
K-means Non-Standardised	2,16,24,29,30,79, MA061, MA064	4,5,14,73,76,132, 135	13,26,31,75,130,159	74,82,83,86	25

The possibility of Case 5 separating out based on weight was further investigated. As this was a seized case, storage conditions may have caused the case to deteriorate at a faster speed and resulted in the chalky appearance. The data was examined using the tablet measurements and standard deviations as shown in 'Chapter 3 - Physical Characteristics'. The results indicated that the diameter and depth of the tablets within Case 5 were slightly larger than the tablets provided by MA Pharmachem (Table 7.6). This resulted in a distinctively heavier weight. In addition, the standard deviations and relative standard deviations of size and weight

indicate a greater variation within the case, compared to the pharmaceutical tablets. This would therefore suggest that the statistical methods were correct in separating out this case from the pharmaceutical tablets.

Table 7.6. Mean weight and size measurements of tablets within Case 5 in comparison to pharmaceutical batches. Information taken from Chapter 3 – Physical Characteristics.

	Diameter (mm)	Depth (mm)	SD of depth (mm)	RSD of depth %	Weight (mg)	SD of weight (mg)	RSD of weight %
Case 5	8.13	3.11	0.057	1.82	216.3	2.60	1.20
MA061	8.08	2.59	0.016	0.62	170.7	1.13	0.66
MA064	8.07	2.65	0.021	0.78	170.3	1.58	0.93

The results of the k-means clustering appeared to be slightly less discerning than the AHC. This is probably because the centroid moves with each addition to the group thus causing it to be heavily influenced by outliers, as explained in section 7.3.2.3. – k-means Clustering.

7.5.3.3 Results of K-Means Clustering of DSC Data

K-means clustering of DSC data was initially performed with five groups requested based on the subjective comparison and then on the nine groups indicated by the AHC.

The results of the five group analysis separated cases 4 and 14 but clustered cases 5 and 25 together (Figure 7.10). The result of the k-means cluster analysis based on the excipients analysed by DSC (Figure 7.10) is compared with subjective clusters in Figure 7.11. As can be seen in the bar charts, ten of the twenty-six cases are clustered differently between the two techniques. It is also worth noting that where cases seem to cluster differently between these two methods, these differences

could also potentially identify further clusters. So, for example, Cases 13, 26, 31 and 159, which formed part of Cluster 1 for the AHC of the DSC data and part of Cluster 3 according to the subjective groupings, still form a cluster together. In addition, they also formed part of a cluster together by AHC analysis of the physical data.

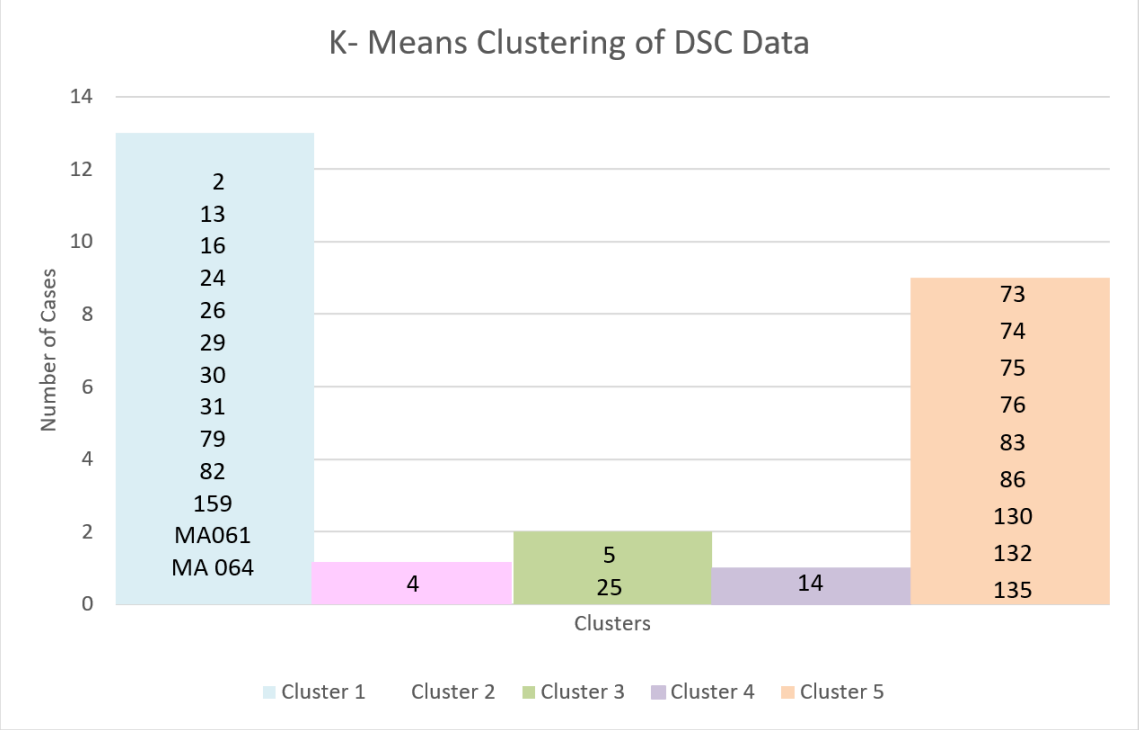


Figure 7.10. K-means clustering of DSC data using MA marked cases.

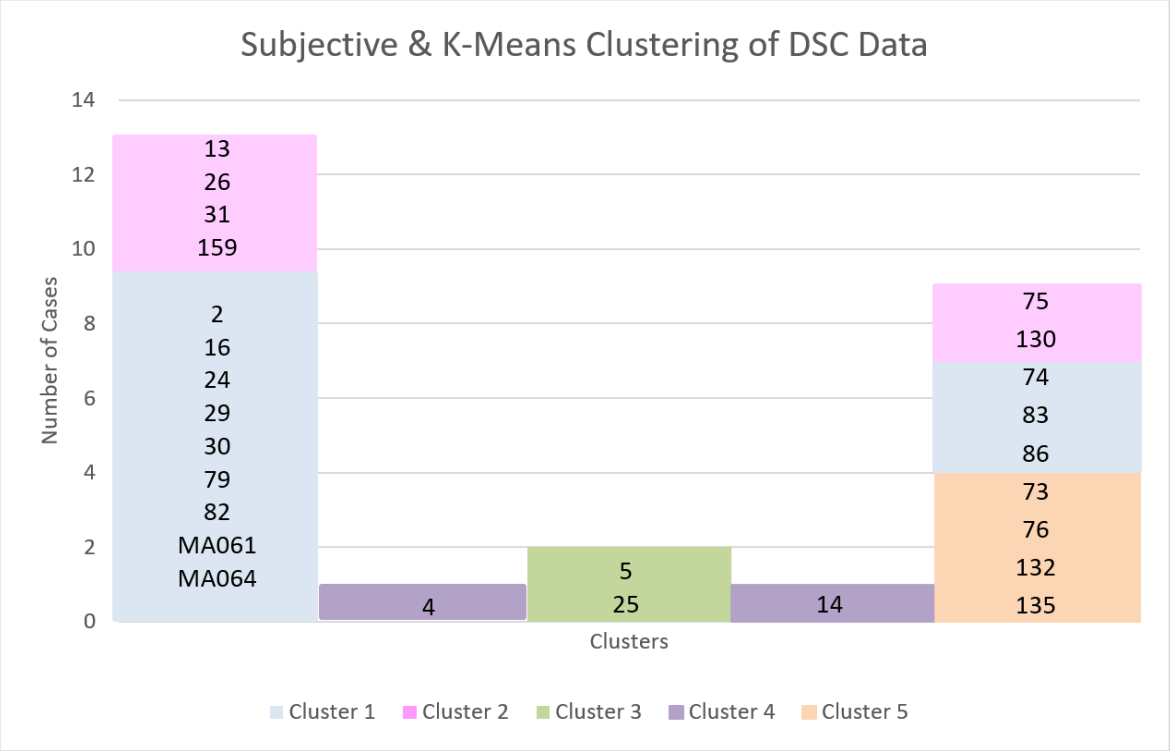


Figure 7.11. K-means clustering of DSC data using MA marked cases. The case numbers have been listed according to groups determined by k-means clustering but have been coloured according to clusters indicated by the subjective groupings.

In order to allow for discrepancies caused through the impact of outliers, as explained in section 7.3.2.2 'Sample Size Test', k-means clustering was also run with 9 clusters as suggested by the AHC dendrogram. The results of this technique meant that the four cases which k-means had clustered with the pharmaceutical tablets but had been subjectively differentiated into Group 2, were again separated (the four cases listed in the pink section of column 1 in Figure 7.11). The k-means analysis distinguishing nine clusters, separated these four cases (13.26.31 and 159) into Cluster 2 (Table 7.7).

The remaining seven cases that had previously clustered with the known pharmaceutical cases were divided between clusters 1 and 5. The reason for this separation is unclear, however the larger the number of groups that are requested in the method, means the more the data will be teased out, to find differences (Table 7.7). The results of this analysis was used in the construction of the heat map (Section 7.4.4).

Table 7.7. The results of K-Means Clustering and AHC of DSC data based on 9 clusters suggested by the AHC dendrogram at a distance of 5.

	K-Means Clustering	Agglomerative Hierarchical Clustering
Cluster 1	2, 29, 30, MA	2, 16, 24, 29, 30 MA
Cluster 2	13, 26, 31, 159	13, 26, 31, 159
Cluster 3	73, 74, 76, 83, 86, 132, 135	73, 74, 76, 79, 82, 83, 86, 132, 135
Cluster 4	75, 130	75
Cluster 5	16, 24, 79, 82	130
Cluster 6	4	4
Cluster 7	14	14
Cluster 8	5	5
Cluster 9	25	25

The sample size is a limitation for the statistical analysis of this project and further work may be required to determine an optimum number of tablets to be analysed. Assessing a higher proportion of tablets in a case improves the reliability of the clustering model but many of the cases recovered by the police contain only small numbers of tablets. This is an exploratory technique however, and there is no hypothesis test so although repeating and increasing the sample size would be helpful, results will largely depend on the tablets analysed.

7.5.4 Creation of Heat Map

The results of the subjective, AHC and k-means clustering techniques were combined and compared using a heat map (Figure 7.12) which identified the number of times different cases clustered together. The results were based on the tests using standardised data and included:

1. Clustering by subjective comparison + drug identification (GC-MS) + quantification (HPLC)
2. Clustering of cases using data from physical analysis (mean and standard deviation of weight) + diazepam (1) quantification by AHC
3. Clustering of cases using DSC generated data by AHC
4. Clustering of cases using data from physical analysis + diazepam (1) quantification by k-Means clustering
5. Clustering of cases using DSC generated data by k-means

Given the three independent data sets (subjective, physical and DSC chemical) and the three types of cluster analysis performed (subjective, AHC and k-means), there was a low chance of multiple grouping if there was no connection between the cases. The probability of two cases clustering repeatedly is illustrated by calculating that if case x was in a given cluster, the probability of case y being in the same cluster would be 1/5 (given 5 clusters are produced, based on the subjective cluster analysis). For every pair of cases, the clustering event will have one or other of two binary outcomes i.e. the cases cluster together (p) or they do not cluster together (1-p) where p is the probability of the cases clustering together by chance alone. The results of one trial do not influence the results of the remaining trials. There are still only two possible outcomes for each trial and the probability of these outcomes remain constant (The Open University, 2009). This is comparable to the binomial calculation demonstrated by independent flips of a coin attaining the same result (Imrey, 2000).

For 3 clustering events (1 with each independent data set) the probability would be calculated using the binomial model:

$$p(k\ successes\ in\ n\ trials) = \binom{n}{k} p^k q^{n-k}$$

Where:

n = number of trials (3)

k = number of successes

p = probability of success in one trial (0.2)

q = 1-p = probability of failure in one trial

n-k = number of failures

Therefore for two cases to cluster together in each of the three events, the probability would be:

3 successes in 3 trials = $\binom{3}{3} 0.2^3 0.8^{3-3}$

3-3 = 0. Anything to power of 0 = 1

$\binom{n}{k} = \frac{n!}{k!(n-k)!}$

So

$\binom{3}{3} = \frac{3\ X\ 2\ X\ 1}{3\ X\ 2\ X\ 1\ X\ 0!}$

As 0! = 1 and 3! / 3! = 1

$(1) 0.2^3 0.8^0$

= 1 x $8x10^{-3}$x1

= 8 $x10^{-3}$

Therefore the probability of 2 cases clustering together on 3 occasions is 8 $x10^{-3}$.

For two cases to cluster together twice in the three trials, the probability would be:

2 successes in 3 trials = $\binom{3}{2}0.2^2 0.8^{3-2}$

$\binom{n}{k} = \frac{n!}{k!(n-k)!}$

So

$\binom{3}{2} = \frac{3\ X\ 2\ X\ 1}{2\ X\ 1\ X\ 1}$

Therefore,

$(3)0.2^2 0.8^1$

= 3 x 0.04 x 0.8

= 9.6 x10^{-2}

Therefore the probability of two cases clustering together on two occasions is 9.6 x10^{-2}.

For two cases to cluster together once in the three trials, the probability would be:

1 success in 3 trials = $\binom{3}{1}0.2^1 0.8^{3-1}$

$\binom{n}{k} = \frac{n!}{k!(n-k)!}$

So

$\binom{3}{1} = \frac{3\ X\ 2\ X\ 1}{1\ X\ 2}$

Therefore,

$(3)0.2^1 0.8^2$

= 3 x 0.2 x 0.64

= 0.384

Therefore the probability of two cases clustering together once during the three trials is 0.384.

These calculations are based on five clusters being produced by each set of data. However, as the AHC and k-means clustering of DSC data generated nine clusters, the probability would have been lower with p = 0.11 and q = 0.89 for those two results.

The probability of the cases clustering together randomly with no connection is therefore small and suggests a potential link between them. A connection between clustered cases could indicate that the cases were produced in the same laboratory or possibly manufactured by the same processes.

The heat map (Figure 7.12) shows that five cases matched with the pharmaceutical tablets on a minimum of four out of five trials. This would suggest that there is a strong connection and cases 2, 16, 24, 29 and 30 could be of pharmaceutical origin. However, the results are not entirely clear cut. Case 79, for example, clusters as many times with Cases 74, 83 and 86 and more times with Case 82 than with the known pharmaceutical tablets. These are the remaining cases that had been subjectively clustered as potential pharmaceuticals. Interestingly, these cases, along with Case 79 are also the cases which were given a reported level of 10 mg of diazepam (**1**) according to hplc analysis but were recognised as different to pharmaceutical tablets, based on DSC thermograms. The origin of these tablets is therefore still uncertain. It is possible that they may be well made counterfeits or degraded pharmaceuticals.

The heat map also identifies potential links between other cases, such as Cases 73, 76, 132, 135, which contain etizolam (**7**). There may also be a possible connection between Cases 13, 26, 31 and 159; and between 74, 83 and 86 all of which cluster in every trial (Figure 7.12).

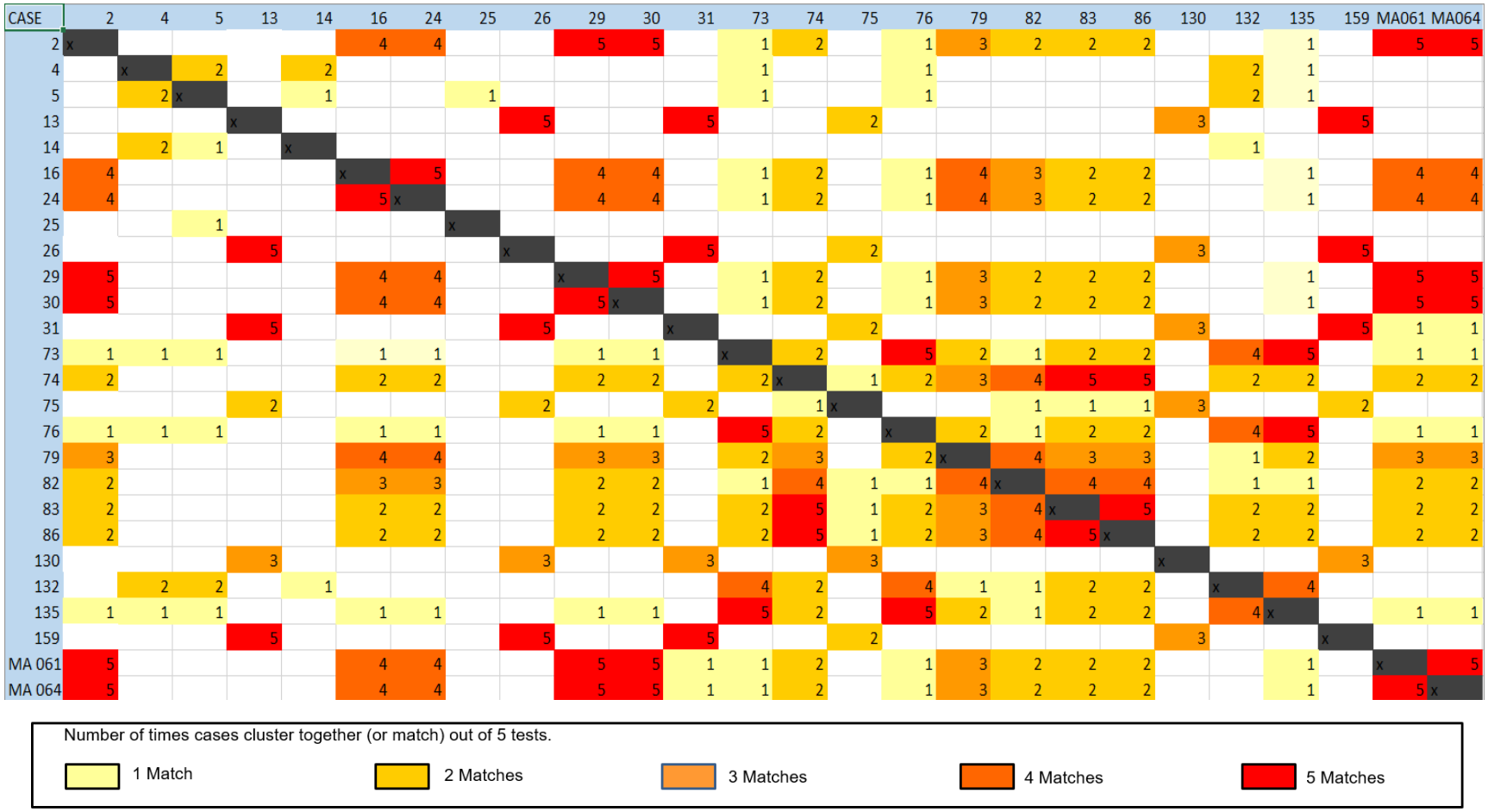


Figure 7.12. Heat map showing a pairwise comparison of all cases. Five different approaches to clustering were carried out and the numbers show the frequency that particular pair of cases were allocated to the same cluster out of a maximum of five clustering attempts. The frequencies are colour coded according to those shown in the legend.

7.6 Conclusion

The aim of the cluster analysis was to clarify the results of the physical and chemical tests on the seized cases, with the intention of distinguishing between the pharmaceutical and illicitly made tablets. In addition, it was hoped that potential links between illicit cases would emerge. Despite the limitations posed by sample size, several groupings were found. For example cases 2, 16, 24, 29, and 30 were identified as potentially being of pharmaceutical origin. In addition, links between illicit cases, such as Cases 13, 26, 31 and 159 were demonstrated by these cases matching in all five tests. However, further background information would be required to support any connection but this information could prove useful to the police.

Ideally a larger sample size would have been beneficial in terms of providing a more accurate mean or centroid on which to calculate the statistics. It could also allow further information to be added to the heat map by performing further analytical tests. This would help to clarify any results. However, the technique of combining all of the analytical and statistical results in a heat map has demonstrated great potential in identifying potential links.

As some of the cases grouped together it indicates that there may be a link between them. It is possible that tablets that grouped together were manufactured together. Some of the seized cases clustered with the pharmaceutical batches a number of times and therefore raises the possibility that they are pharmaceutically made. Pharmaceutical tablets enter the illicit market by a number of methods. A report into countering the problem posed by falsified and substandard drugs, commented that prescription medicines change hands many times between manufacturer and pharmacist, providing many opportunities to divert the tablets (Institute of Medicine, 2014). It was commented that tablets may be stolen at source, from a pharmacy, or by people selling their own, friends or relative's prescription medication, with those stolen closer to source usually providing a much larger quantity for illicit sales. Therefore, illicit cases may be pharmaceutically manufactured but not directly prescribed. It was also noted that many diverted pharmaceutical products

deteriorate over time due to incorrect storage and handling conditions (Institute of Medicine, 2014).

Although the heat map can suggest that potential links exist, it cannot determine if the seized cases containing 10 mg of diazepam (**1**) are of pharmaceutical origin. It could therefore be beneficial to further explore the potential pharmaceutical tablets in order to investigate differences and similarities between them and the genuine pharmaceutical tablets. One method of performing this would be to use supervised statistical methods. By using data produced by tablets known to be illicitly manufactured and known pharmaceutical tablets, a training model can be built. This information can then be used to inform and further test the unknown tablets.

Chapter 8. Statistical Differentiation of Pharmaceutically Manufactured Tablets

8.1 Chapter Summary

In order to further examine the seized cases which contained approximately 10 mg of diazepam (**1**), a smaller test sample was chosen. As the tablets could not be differentiated from the pharmaceutical tablets based on physical characteristics or type and quantity of active drug substance, the data from DSC was analysed by chemometric methods. DSC was chosen because the thermograms generated were largely influenced by the excipient content of the tablets. The data points were explored by principal component analysis and linear discriminant analysis to determine if any differences could be found between the pharmaceutically manufactured tablets and the seized cases.

8.2 Introduction

8.2.1 Tablet Classification

Tablets may be classified based on their physical or chemical properties raising the possibility that legitimately produced tablets can be identified amongst illicit seizures of tablets. Pharmaceutical tablets will possess certain properties such as correct markings and amount and type of active drug substance but well-made counterfeit tablets may also satisfy these requirements so additional aspects of the tablet's contents such as the excipient may help to distinguish pharmaceutical tablets from illicitly made tablets.

8.2.2 Rationale Behind the Statistical Methodology

The aim of this part of the study was to compare the physical and chemical properties of a group of twenty-four illicit tablet batches with MA markings to ten known pharmaceutical tablets to see if there were any illicit tablets that could plausibly have been manufactured by a pharmaceutical company. The first objective

was to separate out all tablets with MA markings then identify and quantify the active drug substance. This used the results of the gas chromatography mass spectrometry (GC-MS) analysis to isolate the cases that contained diazepam (**1**) and high performance liquid chromatography (HPLC) to identify those tablets that contained the pharmaceutical level of around 10 mg of diazepam (**1**).

The second objective was to investigate further those cases that could not be distinguished from the pharmaceutical tablets based on markings and active drug substance alone. The Differential Scanning Calorimeter (DSC) was used to investigate the excipient content of the tablets. Excipients comprise of all the formulated components of a tablet excluding the active drug substance and can include fillers, glidants and binders for example. Each tablet manufacturer chooses the excipients used in their manufacturing process, which means the excipient content of tablets with the same amount and type of active ingredient can differ between companies. Information regarding excipients could therefore help to determine the authenticity of illicit cases because if there is a variation in excipient content between seized cases and pharmaceutical tablets bearing the same marking or logo, this discrepancy could support an argument for the illicit cases having been manufactured in a clandestine laboratory. By analysing the tablets with DSC, a thermal profile is generated which is heavily influenced by the more abundant components within each tablet. In the case of diazepam (**1**) tablets, this is largely the excipients. The thermograms produced can then be compared both visually and by statistical and chemometric analysis.

The third objective was therefore to compare DSC thermograms produced by the illicit and pharmaceutical tablets using multivariate statistical techniques and develop a simple model that could be used to distinguish between MA-marked cases that have been pharmaceutically or illicitly manufactured.

8.2.3 Choice of model

A thermogram is a plot of several thousand heat flow measurements taken at given temperature points between 100 and 250 ^{o}C, from a single observation or tablet. In this study, the heat flow measurement at each temperature is considered an

independent variable and the dependent variable is the binary classification of known pharmaceutical or other manufacturer. The DSC procedure produces a multi-dimensional or multivariable dataset across the whole temperature range examined with each temperature point considered a variable. The final dataset consisted of 7211 variables for each of the 34 observations. However, with such a data set, there is a high degree of correlation between the variables. Correlation between the explanatory variables creates problems for statistical modelling techniques such as regression techniques and linear discriminant analysis, which constructs a discriminant value based on finding significant variables within the dataset that are related to the dependent variable or, in this case, classification group. Correlation can lead to important variables being ignored as insignificant, in favour of alternative, correlated variables. The variables chosen could therefore be defective in representing the true importance of the data, thus creating an unstable and inaccurate model (Hosmer and Lemeshow, 1989). High-dimensionality and multicollinearity can be addressed by using Principal Component Analysis (PCA) to reduce the number of dimensions and produce a number of uncorrelated components.

PCA is an unsupervised statistical method, whereby no information regarding potential groupings is included in the analysis. PCA uses correlation in the independent variables to reduce the dimensionality of the dataset but retains most of the variation of the original dataset within a much smaller set of principal components (PC). This is done by using the data on p variables for n observations so that the first PC (Z_1) is then a linear combination on the variables X_1, X_2 …X_p, which maximizes the variation between the observation such that

Equation 1

$$Z_1 = a_{11}X_1 + a_{12}X_2 \ldots + a_{1p}X_p$$

and

Equation 2

$$(a_{11})^2 + (a_{12})^2 + \ldots (a_{1p})^2 = 1$$

This is repeated for the remaining principal components so that Z_1, Z_2, Z_3… Z_n have zero correlation. A worked example of Principal Component Analysis can be seen in Appendix III.

Once the principal components have been calculated, the number of principal components that account for about 90% of the variation in the dataset (in the study 5 principal components) were used as the variables in the discriminant analysis where they are combined according to equation 1 to calculate the discriminant value (D).

Equation 3

$$D = ((b_1PC1) + (b_2PC2) + (b_3PC3) + (b_4PC4) + (b_5PC5) + c)$$

Where the discriminant function coefficients of the first five principal components of DSC data are represented by $b_{1\ to\ 5}$ and c is a constant associated to the Y-intercept of the regression line (Brown and Wicker,2000).

The first component represents the largest percentage of the variation in the dataset and each subsequent component represents a smaller proportion. Frequently 90% of the data variation resides in the first few components (The Open University, 2007). This means that most of the variation in the dataset can be visualised by plotting the values of the observations on a small number of PCs. Each component, thus allowing visualisation of patterns within a compressed form of the original dataset.

Principal Component Analysis is a widely used technique that is often favoured for exploring spectra because of its ability to compress large data sets by integrating correlated dimensions. Klara Dégardin used Raman Spectroscopy and PCA to firstly identify counterfeit tablets and then to investigate links between the illicit tablets and historical cases. The PCA was particularly beneficial for finding clusters by plotting the principal components to visualise the data (Dégardin *et al.,* 2011). The number of principal components required depends on the data and constituents involved. A recent study by Risoluti only required the first two principal components to be plotted in order to differentiate between two groups of New Psychoactive Substances (NPS) (Risoluti *et al.,* 2016). However, it has been noted that only a portion of the variation can usually be visualised with PCA alone (NicDaéid and Waddell, 2005). Dégardin

used PCA to gain an initial insight into potential clusters of pharmaceutical and illicit tablets before following it up with supervised statistical methods (2016).

Once the dataset has been compressed, the principal components can be used as surrogate explanatory variables in follow-on modelling procedures such as linear discriminant analysis (LDA). LDA is a method that uses low-dimensional data to highlight differences between subgroups. Information regarding the properties of the subgroups, whose classification is already known, is then used to create allocation rules which enable predictions to be made regarding the groupings of new observations (The Open University, 2007; Brown and Wicker,2000).

The supervised technique of Linear Discriminant Analysis (LDA) was used by Johnston and King (1998), to detect links between heroin samples by analysing the levels of alkaloids and adulterants in the sample. This ultimately led to some success in predicting their country of origin. LDA was also applied to discrimination of young drug and fibre type cannabis plants according to chemical composition of their leaves as identified by GC-MS. This technique classified observations correctly with 89% accuracy (Broséus, Vallat and Esseiva, 2011). Similarly, LDA with leave one out cross validation was used to classify saffron into its geographical area of origin based on chemical constituents identified through HPLC analysis. Although it proved more difficult to distinguish between geographical areas that were closer together which, increased the misclassification rate (D'archivio *et al.,* 2016). Lesiak (2014) used LDA to distinguish between Mitragyna speciosa and other plant species based on the mass spectral data generated by Direct Analysis in Real Time - Mass Spectrometry (DART-MS), with 99% accuracy.

In a study investigating counterfeit medicines, Dégardin used a variety of statistical techniques for distinguishing between samples. This included PCA and Receiver Operating Characteristic (ROC) curves. However, the most effective analysis resulted from studying a variety of information gained from all available resources, including police reports, in order to identify potential links (Dégardin *et al.,* 2011).

In this chapter, I describe how data from DSC thermograms were used to develop an allocation rule to distinguish between pharmaceutical and non-pharmaceutical tablets using a combination of PCA and LDA.

8.3 Aim of Statistical Model

The aim of this section was to devise a model that could distinguish between illicit cases of MA tablets and known, pharmaceutically manufactured 10 mg diazepam tablets. This first step was to examine the tablet type and quantity of diazepam (**1**) present, allowing classification of those tablets that did not contain 10 mg of diazepam (**1**) as non-pharmaceutical. The second step used data from DSC thermograms and linear discriminant analysis to create a model that distinguishes pharmaceutical tablets with 10 mg of diazepam (**1**) from illicit, 10 mg diazepam (**1**) tablets.

8.4 Analytical Procedure Employed

8.4.1 The MA/D10 Test Group

Visual examination of the tablets revealed that twenty-four of the sixty-eight illicit cases bore the markings MA/D10. The number of cases of this design is illustrated in Figure 8.1. These markings relate to a recognised pharmaceutical company, MA Pharmachem, which provided 10 mg diazepam (**1**) tablets for the United Kingdom. Although MA Pharmachem no longer produce diazepam (**1**) tablets, these tablets may have still been legitimately available at the time of seizure due to the length of their shelf life after manufacture.

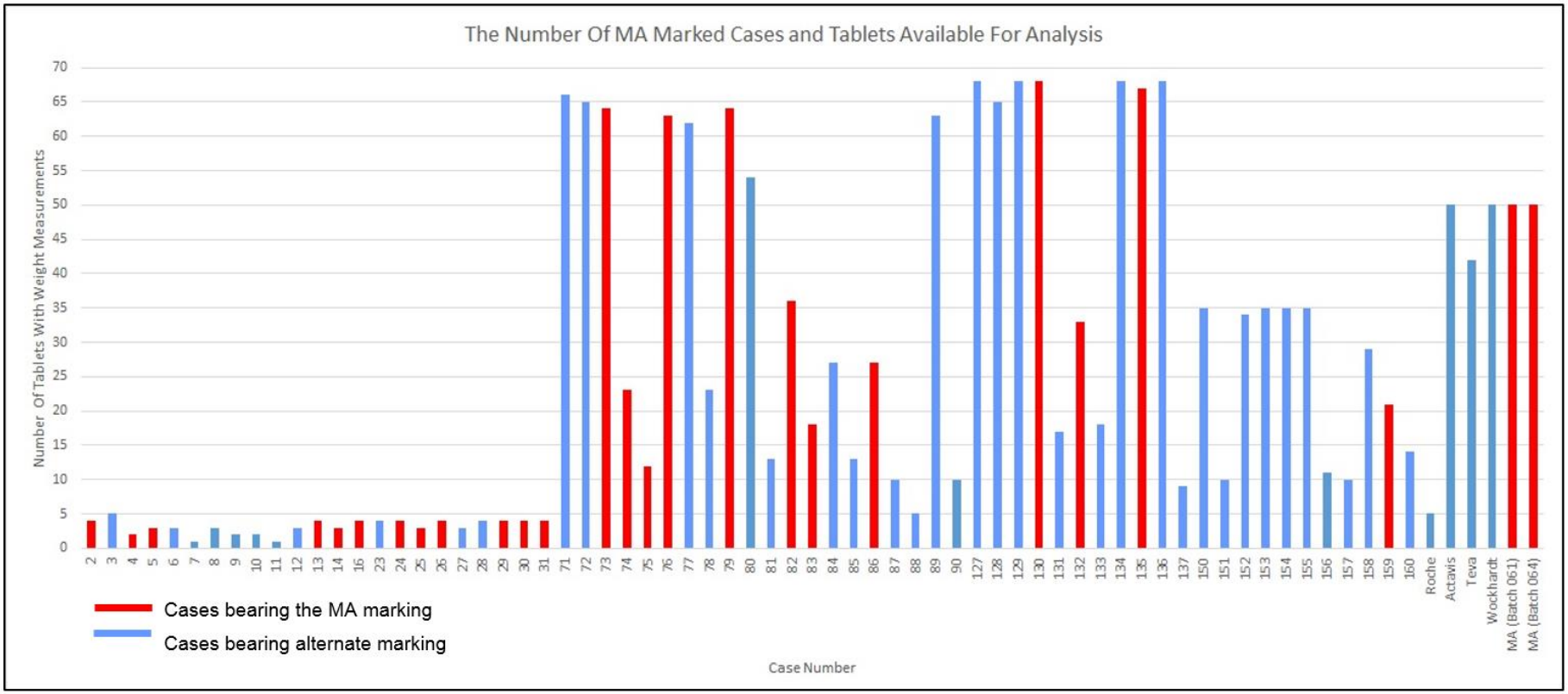


Figure 8.1. Bar chart showing the cases marked MA and D/10 highlighted in red, and the amount of tablets within the cases.

Twenty-four illicit cases had the MA marking and relevant data regarding tablet weight and specifications was provided by the pharmaceutical company, along with two batches of pharmaceutically manufactured tablets for comparative purposes. The statistical analysis used physical and chemical data from all of the twenty-four illicit cases containing tablets marked MA and D/10, to assess whether these cases had the same properties as the pharmaceutically manufactured tablets.

8.4.2 Pharmaceutical and Non Pharmaceutical Tablets

The twenty-four illicit cases of MA tablets could have either been produced illicitly or pharmaceutically manufactured but then diverted into the illegal supply chain. For the purposes of this project, 'pharmaceutically manufactured' refers to tablets that were produced legitimately for the UK market. Illicit tablets may have been imported from other sources via the internet and could include either clandestine laboratories or pharmaceutical companies in other countries. However, if the tablets did not meet the manufacturers' specifications and therefore were not believed to be legitimately manufactured by pharmaceutical companies (MA Pharmachem in this instance) for UK consumption, the tablets were deemed as illicit.

8.4.3 Separation of Illicitly Manufactured Tablets

8.4.3.1 Identification of the Active Drug Substance by GCMS

Chemical analysis by Gas Chromatography-Mass Spectroscopy (GC-MS) showed that not all the twenty-four illicit cases marked MA/D10 contained diazepam (**1**). Six cases contained phenazepam (**16**) or etizolam (**7**) and were automatically deemed illicitly manufactured tablets without consideration of properties since these active drug substances are not licenced within the UK. These cases were included in the remainder of the study as examples of illicit tablets. The remaining 18 cases contained diazepam (**1**) and were then analysed by HPLC to ensure the quantity of active drug substance was consistent with the manufacturer's specification (Figure 8.2).

8.4.3.2 Quantification of Diazepam by HPLC

High Performance Liquid Chromatography (HPLC) was used to quantify the diazepam (**1**) content in the remaining 18 illicit cases. Six of the illicit cases contained over 20 mg of diazepam (**1**) and two contained approximately 8 mg. The tablets in these cases did not meet the manufacturer's specification for quantity of diazepam (**1**) and were classified as non-pharmaceutical, leaving ten cases with the correct type and quantity of active drug substance and the potential to have been pharmaceutically manufactured (Figure 8.2).

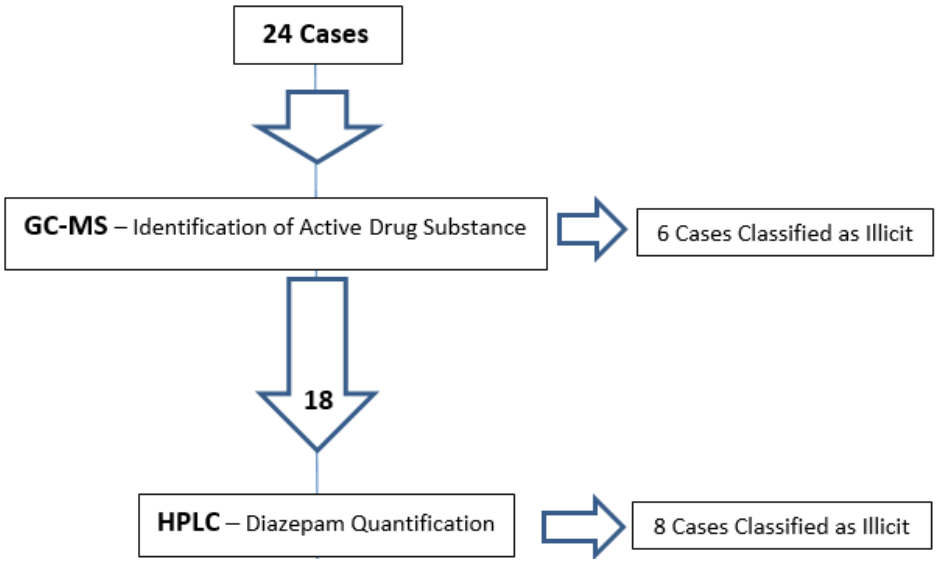


Figure 8.2. Flow chart showing the separation of cases that had been illicitly manufactured.

The latter group of illicit tablets were labelled the 'unknown' group because they were illicit seizures but their diazepam (**1**) content was indistinguishable from pharmaceutical tablets and based on this information alone, they could have been either illicitly manufactured or pharmaceutically manufactured but diverted into the illicit diazepam (**1**) market.

8.4.3.3 Excipient Analysis using DSC

The next step was to explore the excipients in the illicit cases to identify those that corresponded with the pharmaceutical tablets. As each tablet weighed approximately 270 mg and contained around 10 mg of diazepam (**1**), the bulk of the tablets were comprised of excipients. Excipients include all of the constituents added to a tablet apart from the active drug substance. Pharmaceutically manufactured diazepam (**1**) tablets in the UK contain a large quantity of lactose that increases the tablet size and makes it easier to handle. Although other excipients, such as lubricants and binders are also present, the large quantity of lactose is a strong influence on Differential Scanning Calorimetry.

Results produced by the Differential Scanning Calorimeter (DSC) for this project, demonstrated that individual cases could be differentiated based on excipient content (Chapter 6 Differential Scanning Calorimetry). DSC measures the difference in heat flow between a sample and a reference pan. The heat flow is plotted against temperature to create a thermogram, as demonstrated in Figure 8.3 (also see Chapter 6). A thermogram was produced for each of the twenty-four cases of illicit tablets marked MA and for 10 pharmaceutical MA tablets.

The temperature on the thermograms ranged between 22 – 240 ^{o}C (Figure 8.3) but as the lower temperatures (<100 ^{o}C) reflect solvent and water loss from the tablet rather than the thermal characteristics of the substances present, this area of the thermogram was removed prior to the statistical analysis. Likewise, degradation of the sample dominated higher temperatures so did not represent particular characteristics of the substances present and were not included in the analysis. Therefore, the statistical analysis used only the temperature range from 100 - 220 ^{o}C (Bibi *et al.,* 2015). Each thermogram comprised thermal flow measurements at 7211 temperature points.

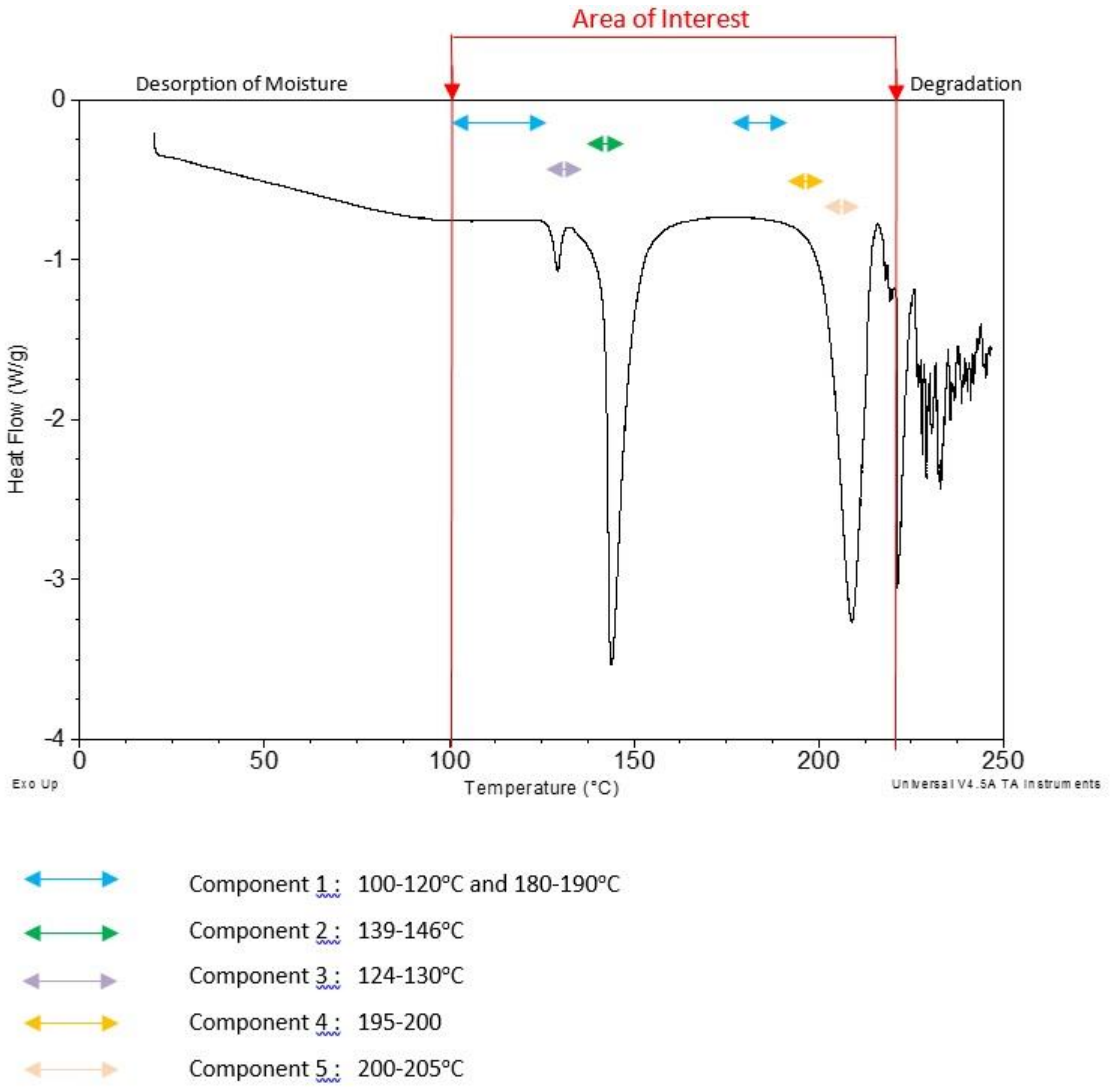


Figure 8.3. DSC Thermogram indicating the temperature regions dominating each of the first five principal components.

8.4.3.4 Reducing DSC dataset dimensionality with Principal Component Analysis (PCA)

Examining the type and quantity of active drug substance had left ten illicit cases that were indistinguishable from pharmaceutical tablets so the next phase of the study was to develop a linear discriminant analysis (LDA) to distinguish between these two groups using DSC data that reflected the excipient content of the tablets. DSC analysis produced a high dimensional, multivariate dataset however, having such a large number of variables leads to problems of multicollinearity and overfitting of statistical models. Having correlated independent variables in an LDA model limits

the discriminatory power of the variables leading to an unstable model. An ideal model is a parsimonious model with a small number of highly discriminating variables, therefore in order to achieve this, principal component analysis (PCA) was used to render the 7211 temperature variables in the DSC dataset to a small number of uncorrelated components (Garson, 2012). Therefore, Principal Component Analysis (PCA) was performed on the thermal data produced by DSC, using the "R" statistical programming language (The R Foundation, 2017). Thirty-four principal components were generated, with 79% of the variation contained in the first three components. Ideally, the analytical variation should be higher but 90% variation was not reached until the fifth component. Five components were therefore used as explanatory variables in the LDA model but since five dimensions are not easily visualised, only the first three dimensions (79%) of the variation are shown in the PCA plots (Figure 8.5). The variances attributed to the first five principal components are shown in Table 8.1. The principal components that dominated different regions of the DSC thermograms are shown in Figure 8.3.

Table 8.1. The variance in the first five principal components.

Component	% Variance	Cumulative %
1	57.09	57.09
2	14.16	71.26
3	7.55	78.81
4	5.98	84.79
5	5.79	90.58

8.4.3.5 Visualisation of the Results of Principal Component Analysis

Principal Component Analysis (PCA) was carried out on the DSC dataset that incorporated all illicit cases with MA markings and the ten known pharmaceutical tablets. The results of the PCA on all cases were visualised using Miner 3D (Figure 8.4). This visualisation of the dataset shows the first two principal components of the DSC data and the quantity of the active drug substance diazepam (**1**), present in the tablets as measured by HPLC as the third axis (Chapter 5).

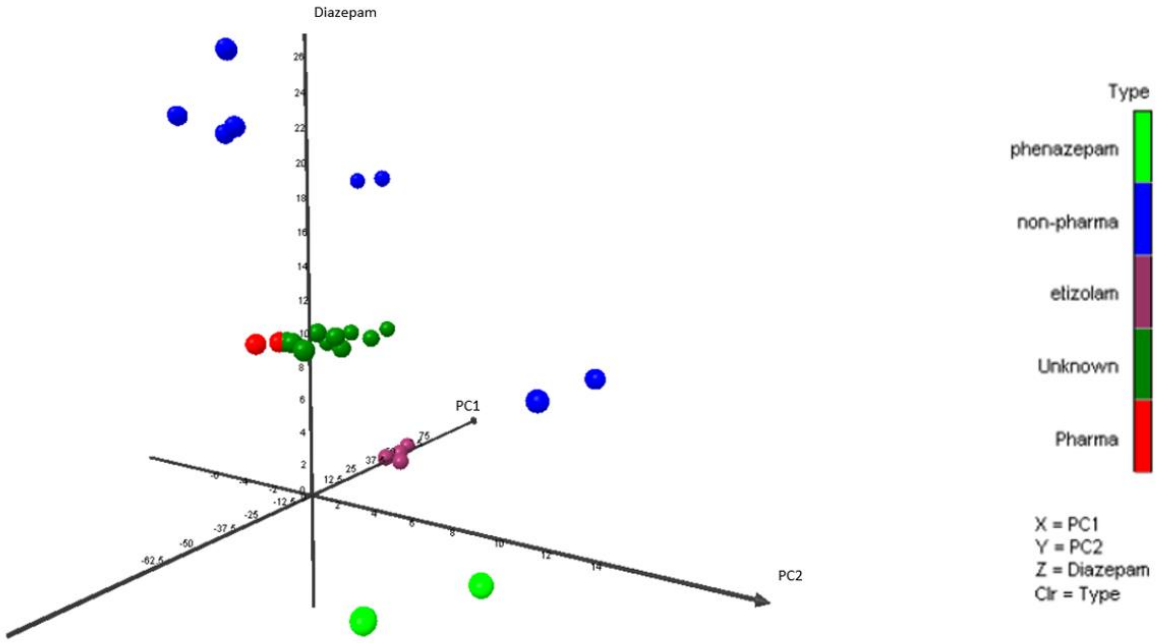


Figure 8.4 Miner 3D image showing PCA results of the DSC data. The first two principal components plotted against the level of diazepam (1). Groupings can be identified according to the active drug substance (identified by GC-MS and colour-coded) and the amount of diazepam (1), where present (quantified by HPLC). The tablets labelled as 'Non-Pharma' contain levels of diazepam (1) that do not meet pharmaceutical specification. Tablets containing a different active drug substance are labelled 'phenazepam' (16) or 'etizolam' (7) according to the GC-MS results. Unknown tablets (illicit tablets with 10 mg of diazepam (1)) and genuine pharmaceutical tablets are also indicated.

There are clear groupings in the illicit tablets (Figure 8.4) depending on the diazepam (**1**) content and the active drug substance. The genuine pharmaceutical tablets are illustrated in red as a separate group. However, these groups are not just distinguishable by their active drug content. The tablets can also be separated by their values on PC1 and PC2 from the DSC data. For example, the PC1 values range between 79 to 113 for tablets containing etizolam (**7**), -100 to -47 for tablets containing phenazepam (**16**) and between -110 to -25 for pharmaceutical tablets, while the PC2 values range between -4.5 to +5 for tablets containing etizolam (**7**), 65 to 80 for tablets containing phenazepam (**16**) and between -30 to +2.5 for pharmaceutical tablets. This separation indicates variation in the excipient content as well.

Two groups could not be distinguished using active ingredient content alone; these groups were named unknown tablets and genuine pharmaceutical tablets. As the level of diazepam (**1**) present in the pharmaceutical tablets and unknown tablets was similar, the quantity of drug substance was no longer useful in discriminating between the groups of interest in this analysis but these two groups clustered separately on the PC1 and PC2 axes from DSC analysis which represented the excipient content. Separation between these two groups was clearer if the DSC data were plotted on the first three principal components (Figure 8.5).

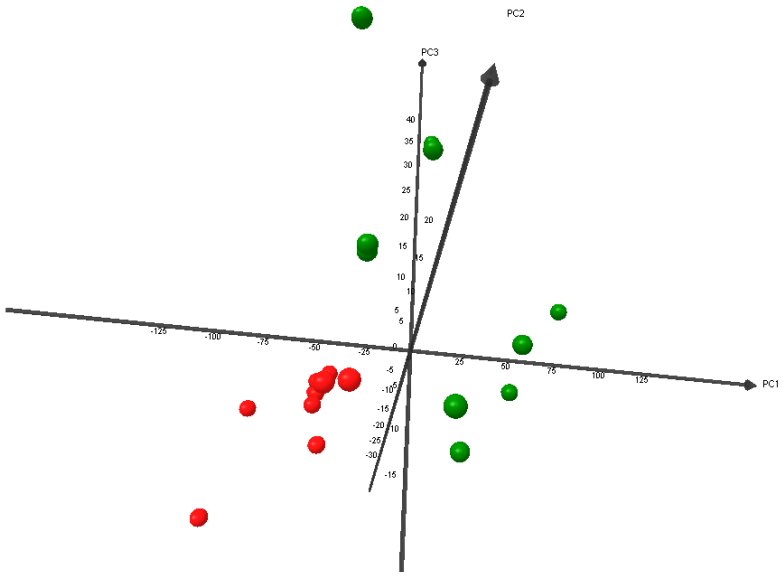


Figure 8.5. Miner 3D image showing separation between the pharmaceutical (red) and unknown (green) cases based on the first three principal components of the DSC data.

The subjective impression of the image illustrated in Figure 8.5, shows that there is separation between the two groups, based on the DSC data. Therefore, the hypothesis that the two groups of tablets could be distinguished based on their DSC thermogram alone, was investigated further using linear discriminant analysis.

8.4.3.6 Application of Linear Discriminant Analysis

The next step was to separate the pharmaceutical tablets from the unknown tablets. A variety of statistical techniques are available for classification purposes, including binary logistic regression. However, Linear Discriminant Analysis (LDA) is generally simpler to use and does not undergo the level of iterations that slow down the calculations in the logistic regression. LDA is also more precise in its results as long as the assumption of normality is met (Tufféry and Tuffery, 2011).

The dataset is first split into two subsets; a training set and a test set. To discriminate between groups of observations, an LDA model is produced using samples from the training set where the classification is known and a classification rule can be formulated. The quality of this classification rule is assessed by applying it to the test set and recording the number of observations in the test set that are classified correctly

The group used for testing the model was comprised of data from two randomly chosen cases containing illicit tablets and one tablet taken from each of the two batches provided by MA Pharmachem. The remaining illicit cases and an equal number of pharmaceutical tablets comprised the training set. The stratagem used to distinguish between illicit cases and those with the potential to have been pharmaceutically manufactured, is demonstrated in Figure 8.6.

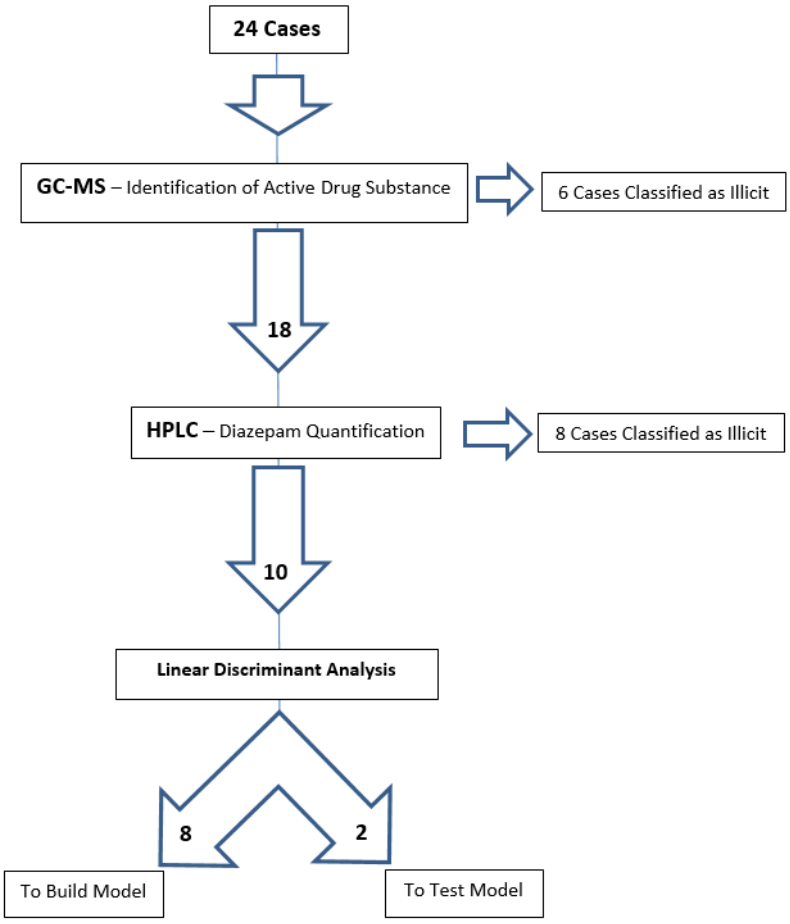


Figure 8.6. The stratagem for discriminating between tablets which had been illicitly and pharmaceutically manufactured. The numbers of illicit tablets used to build and test the model were matched with pharmaceutical tablets.

The independent variables used for the linear discriminant analysis were the first five components of the PCA carried out on the DSC data. Monfreda (2015) chose to use the first ten principal components for analysis into cocaine and gave equal weighting to the data generated by the alkaloid and residual solvent. For this study, five components were used for Model 1 because this contained 90% of the variation from

the original dataset. A second model using only PCs 1, 2 and 5 was also tested as an alternative. The second model was developed because two PCs were found to have no significant discriminatory power (Table 8:10) and were therefore removed to form a more parsimonious model (model 2). This is discussed in Section 8.4.6 'Suitability of Data'.

The analysis was performed with principal components that were both weighted and unweighted by the proportion of variance determined by the PCA. This was to reflect the difference in discriminating power of each of the components. However, although both methods were attempted with only slight differences in the figures, the results were the same and so only the results from the weighted data is shown below. The weighting was applied by multiplying the principal component values by their % variation prior to using them as independent variables for the LDA.

8.5 The Statistical Model Applied

8.5.1 Aim of the Analysis

The aim of the linear discriminant analysis was to see if the DSC thermograms and hence the excipient content of the group of unknown tablets could be distinguished from pharmaceutically manufactured tablets. If unknown tablets were indistinguishable from the pharmaceutical tablets, this could indicate that pharmaceutical tablets had been diverted into the illicit market. If the groups were distinct, a number of possible explanations could exist:

1. Unknowns were not pharmaceutically manufactured
2. Unknowns were pharmaceutically manufactured with a slight difference in formulation
3. Unknowns were pharmaceutically manufactured but the formulation had deteriorated over time.

8.5.2 Results of Linear Discriminant Analysis

Linear discriminant analysis creates a discriminant function (D), which is a weighted combination of the independent variables in the dataset, in this case, PC1 – PC5 of the DSC data variables. A value (D) for each observation (tablet) on the discriminant function is determined and based on this value, an allocation rule was created to separate the tablets which share the characteristics of the pharmaceutical tablets and those which do not. The discriminant values for each observation in the training dataset were determined according to equation 1, where $a_{1 \text{ to } 5}$ are the discriminant function coefficients for the first five principal components of the DSC data and c is a constant related to the Y-intercept of the regression line (Brown and Wicker,2000).

Equation 3

$$D = ((b_1PC1) + (b_2PC2) + (b_3PC3) + (b_4PC4) + (b_5PC5) + c)$$

In terms of the models used for this project, the equations translate as:

Model 1

D = ((PC1 x 0.031) + (PC2 x 0.089) + (PC3 x -0.17) + (PC4 x 0.107) + (PC5 x 0.035) + 0.573).

Model 2

D = ((PC1 x 0.023) + (PC2 x 0.086) + (PC5 x 0.027) + 1.378).

8.5.3 Allocation Rules

The discriminant function values (D) were then used to establish an allocation rule. There are a number of ways this allocation rule can be calculated but three simple approaches were compared in this study. The first method used the average of the central D values for each group as a cut-off point. This is the default method used in SPSS and the independent variables are weighted such that the average for the centroid values of the two groups is zero.

Secondly, 95% confidence intervals for the mean D value for each group were calculated and a cut-off point for group allocation was determined as the midpoint between the lower 95% confidence limit of the mean D value for outcome = unknown and the upper 95% confidence limit of the mean D value for outcome = pharmaceutical (Figure 8.7).

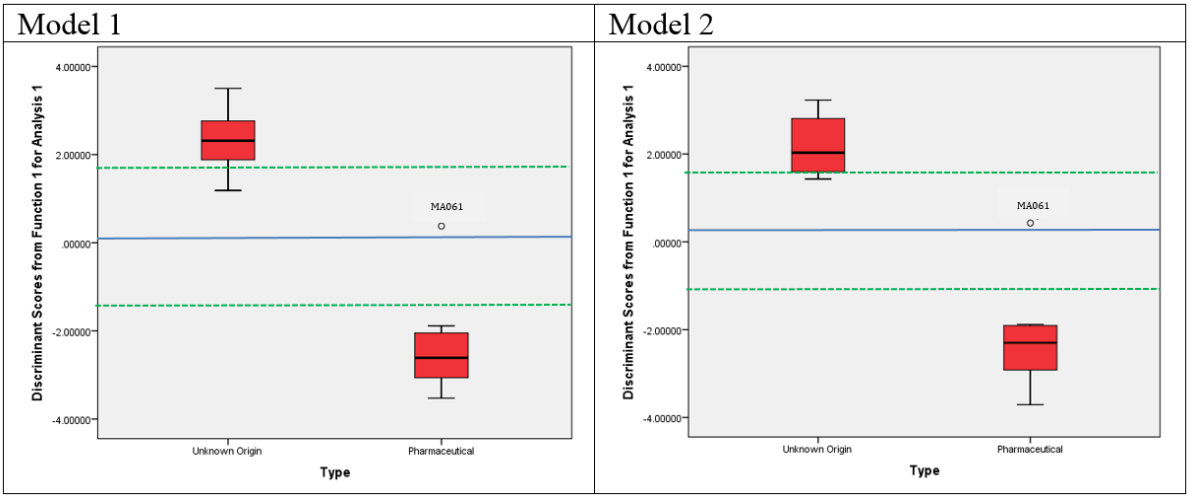


Figure 8.7. Box plot of the D values. The green dotted lines indicate the lower boundary of the 95% confidence interval for the mean D value for the unknown group and the upper boundary of the 95% confidence interval for the mean D value for the pharmaceutical group. The blue line indicates the average value for these boundaries and the cut-off lines at 0.22 for model 1 and 0.23 for Model 2.as blue lines.

The final allocation technique involved the creation of a Receiver Operating Characteristic curve (ROC), which plots sensitivity vs 1-specificity from the predicted allocations for various values of D. A cut-off value of D was selected that maximized the sensitivity and specificity of the predicted allocations.

8.5.3.1 Investigation of Centroids

Using discriminant analysis on SPSS, the default setting gives weight to the centroids creating a midpoint of “0”, which is used as the demarcation between the different types of tablets. Cases that are evaluated with a discriminant function value above “0” are classified as being of unknown origin and those which are evaluated below “0” are classified as being of pharmaceutical origin.

Allocation rule 1:

$$\text{If D} > 0 \begin{cases} classify\ as\ unknown \\ otherwise\ pharmaceutical \end{cases}$$

The discrimination value of “0” misclassified one of the known pharmaceutical tablets as being of unknown origin, thus resulting in a sensitivity value of 0.875 and a specificity of 1. The closer the sensitivity and specificity are to 1 reflects the closer the results are to the original classification. The results of models 1 and 2 are shown in Figure 8.8 and the corresponding data in Table 8.2.

8.5.3.2 Investigation into Confidence Intervals

The 95% confidence intervals were calculated for the unknown cases and the pharmaceutical tablets using SPSS. The midpoint between the lower confidence interval of the D value of the data for the unknown cases and the upper confidence interval for the pharmaceutical data was taken as the demarcation value.

Allocation rule 2 (model 1):

$$\text{If D} > 0.22 \begin{cases} classify\ as\ unknown \\ otherwise\ pharmaceutical \end{cases}$$

Allocation rule 2 (model 2):

$$\text{If D} > 0.23 \begin{cases} classify\ as\ unknown \\ otherwise\ pharmaceutical \end{cases}$$

The use of the confidence intervals in determining a cut-off point resulted in one of the known pharmaceutical tablets being misclassified as unknown. This again resulted in a sensitivity value of 0.875 and a specificity of 1. The results are shown in Figure 8.8 and the corresponding data in Table 8.2.

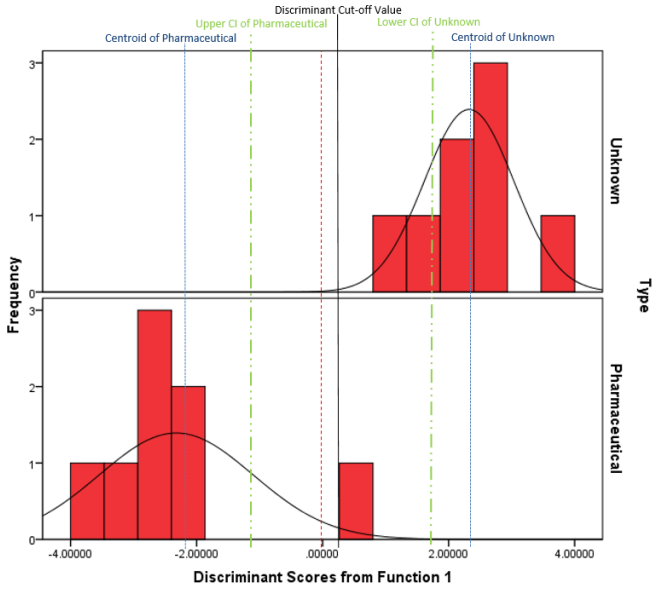


Figure 8.8a

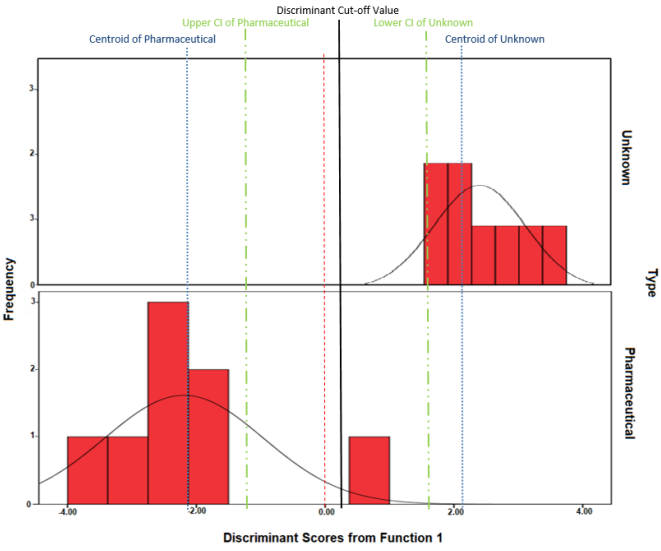


Figure 8.8b

Figure 8.8. Histograms showing the separation of the unknown cases from the cases that were known to be pharmaceutically manufactured and their normal distribution curves. The two blue lines identify the centroids for each type and the default discrimination line of 0, midway between the centroids, which was used in method 1 and is marked by the red dotted line. The dotted green lines represent the 95% Confidence Interval boundaries used to determine the cut-off point used to separate the two types of tablet in method 2. The cut-off point is shown as the black line at 0.22 for Model 1 (Figure 8.8a) and 0.23 for Model 2 (Figure 8.8b).

Table 8.2. Results of allocation rules. The original classifications indicate whether the tablet was taken from one of the seized cases (unknown origin) or provided by the pharmaceutical manufacturer. Under each model description is the result of the statistical groupings and shows that one pharmaceutical tablet was misclassified using the first two methods but the ROC curve correctly identified all of the tablets. Model classification uses the data in the dataset used to train the LDA model.

		Model 1		Model 2	
	Original Classification	Unknown Origin	Pharmaceutical	Unknown Origin	Pharmaceutical
Method 1 Centroids	Centroids of Groups	2.325	-2.325	2.192	-2.192
	Weighted Centroid Average	If D>0 classify as unknown otherwise pharmaceutical	If D<0 classify as pharmaceutical otherwise unknown	If D>0 classify as unknown otherwise pharmaceutical	If D<0 classify as pharmaceutical otherwise unknown
Model Classification	Unknown Origin	8	1	8	1
	Pharmaceutical	0	7	0	7
	Sensitivity	0.875		0.875	
	Specificity	1		1	
Method 2 Confidence Intervals	95% Confidence Intervals	Upper = 2.92 Lower = 1.73	Upper = -1.30 Lower = -3.35	Upper = 2.77 Lower = 1.61	Upper = -1.16 Lower = -3.22
	Confidence Limit	If D>0.22 classify as unknown otherwise pharmaceutical	If D<0.22 classify as pharmaceutical otherwise unknown	If D>0.23 classify as unknown otherwise pharmaceutical	If D<0.23 classify as pharmaceutical otherwise unknown
Model Classification	Unknown Origin	8	1	8	1
	Pharmaceutical	0	7	0	7
	Sensitivity	0.875		0.875	
	Specificity	1		1	
Method 3 ROC Curve	ROC Cut-Off Value	If D>0.78 classify as unknown otherwise pharmaceutical	If D<0.78 classify as pharmaceutical otherwise unknown	If D>0.93 classify as unknown otherwise pharmaceutical	If D<0.93 classify as pharmaceutical otherwise unknown
	Area Under Curve	1.00	1.00	1.00	1.00
Model Classification	Unknown Origin	8	0	8	0
	Pharmaceutical	0	8	0	8
	Sensitivity	1		1	
	Specificity	1		1	

8.5.3.3 Receiver Operating Characteristic curve (ROC) Analysis

The final allocation rule used a ROC curve, which operates a balance between sensitivity and specificity, to determine a cut-off point between the two groups and the discrimination value is chosen as the point that maximises both sensitivity and specificity. According to the dataset used to train the LDA model, this value of D was D = 0.78 for Model 1 and D = 0.93 for Model 2. A lower cut-off value would reduce the specificity of the model and a higher cut-off value would decrease the sensitivity. In this case, the sensitivity is the true classification rate for pharmaceutical tablets and specificity is the true classification rate for illicit/unknown tablets. Therefore,

Sensitivity =

$$\frac{no\ correctly\ classified\ (True) as\ Pharmaceutical\ (TP)}{(no\ correctly\ classified\ (True) as\ Pharmaceutical\ (TP) + no\ incorrectly\ classified\ (False)\ as\ unkNown(FN))}$$

Specificity =

$$\frac{no\ correctly\ classified\ (True)\ as\ unkNown\ (TN)}{(no\ correctly\ classified\ (True)\ as\ unkNown\ (TN) + no\ of\ incorrectly\ classified\ (False)\ as\ \ pharmaceutical(FP)}$$

A ROC curve plots sensitivity against 1-specificity to produce a curve. The closer this is to the top left of the plot, the higher the proportion of true positive results and hence the more accurate the calculation. If the curve lies close to the diagonal line (Figure 8.9), the classification is no better than random allocation of the tablets to the two groups (Altman and Bland, 1994). This means that the closer the area under the curve is to 1, the closer the LDA model classification is to a perfect classification according to the original classification of pharmaceutical and unknown. The ROC analysis was carried out in SPSS and the results for both models (1 and 2) indicated that this was a perfect classification, with no false positives being recorded. The results are shown in Table 8.2 and Figure 8.9.

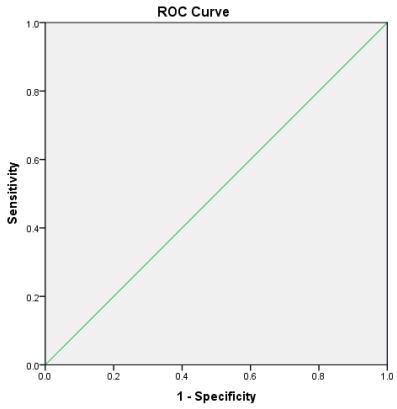


Figure 8.9. The ROC curve produced by the analysis of the principal components of the DSC data. The result was the same for both models 1 and 2.

Two validation methods were employed to assess the performance of the models.

8.5.4 Validation of the Statistical Technique

8.5.4.1 Validation by Leave-one-out analysis

Validation was performed in order to ensure the model was fit for purpose by testing whether the model predictions were reliable. Validation of the model was performed using 'Leave-one-out' analysis. This technique runs the application *n* times using *n*-1 observations from the dataset each time and then the model is tested on the excluded observation as a form of cross-validation. After all the *n*-1 iterations of the model, each observation has been used once to test the model and *n*-1 times to train the model. Each of the samples are therefore used for testing the classification capabilities of the model.

The results of the validation show that although all the unknown cases were classified by the model in accordance with the original classification during the building process and one pharmaceutical was incorrectly classified as being of unknown origin, an additional unknown case was classified as being pharmaceutical during the cross-validation (Table 8.3). The validation was performed using both models 1 and 2, producing identical results. The Leave-One-Out Cross Validation

(LOOCV) was performed by SPSS using the default mean centroid allocation rule which has the cut-off D>0 for unknown and D<0 for pharmaceutical. Options for changing the cut-off to test the other two allocation rules were not available in SPSS and this therefore made an impact on the results since the third allocation rule produced a more reliable classification on the data used.

Table 8.3. Classification and Cross-Validation Results using both models 1 and 2.

		Predicted Group Membership		
	Type	Unknown Origin	Pharmaceutical	Correct Classification (%)
Original	Unknown Origin	8	0	93.8
	Pharmaceutical	1	7	
Cross-Validated	Unknown Origin	7	1	87.5
	Pharmaceutical	1	7	

8.5.4.2 Validation of models using test sample

Prior to developing the LDA models, the dataset was split into a training dataset for training the model and a testing dataset to test the predictive quality of the model. The model for the LDA had been trained using all of the unknown cases except for two chosen at random (Cases 2 and 29) and two pharmaceutical tablets also taken at random (MA064 and MA061-30). These reserved cases of unknown tablets and two pharmaceuticals, were used for testing the two models. Ideally a larger number of samples would have been used for the test but this was not possible given the small number of tablets available for the dataset.

8.5.4.2.1 Centroids

The SPSS default of 0 was used to analyse all of the four test cases, using both models 1 and 2. All of the test samples were correctly identified using Model 1. However, one pharmaceutical tablet was misclassified as being of unknown origin by using Model 2. In addition, all of the tablets that did originate from an unknown source were classified as being from the unknown group. Since there were only

four samples in the test dataset, the number of observations is too small to calculate separate sensitivity and specificity. The results are shown in Table 8.4.

Table 8.4. Results of the four test cases using discriminant analysis with centroids and the SPSS default discriminant value of 0.

		Model 1		Model 2	
	Original Classification	Unknown Origin	Pharmaceutical	Unknown Origin	Pharmaceutical
Unknown Origin	Classification	2	0	2	1
Pharmaceutical	Classification	0	2	0	1

8.5.4.2.2 Confidence Intervals

Calculations of the midpoint between the upper 95% confidence interval of the pharmaceutical data and the lower 95% confidence interval of the unknown data, meant that the cut-off point moved only fractionally from the default point of 0 to 0.12 for Model 1 and to 0.21 for Model 2. This resulted in all of the test samples being correctly classified using Model 1, and one pharmaceutical tablet being misclassified as unknown, using Model 2. However, all the tablets in the dataset that were known to be of unknown origin were correctly classified using this allocation rule (Table 8.5).

Table 8.5. Results of Discriminant Analysis using 95% confidence interval boundaries using the data from the four test cases and the training samples.

		Model 1		Model 2	
		Unknown Origin	Pharmaceutical	Unknown Origin	Pharmaceutical
Method 2 Confidence Intervals	95% Confidence Intervals	Upper = 2.54 Lower = 1.37	Upper = -1.13 Lower = -2.78	Upper = 2.22 Lower = 1.26	Upper = -0.85 Lower = -2.64
	Confidence Limit	If D>0.12 classify as unknown otherwise pharmaceutical	If D<0.12 classify as pharmaceutical otherwise unknown	If D>0.21 classify as unknown otherwise pharmaceutical	If D<0.21 classify as pharmaceutical otherwise unknown
Classification	Unknown Origin	2	0	2	1
	Pharmaceutical	0	2	0	1

The results generated by the two models were slightly different. Model 2 only used three of the five principal components, so it may be that although components 3 and 4 did not contribute as much to the final result, it still made an impact on the final classification.

8.5.4.2.3 ROC Curve

The allocation rule based on the ROC curve correctly classified all cases (Table 8.6). Both models 1 and 2 produced an area under the curve of 1, showing no discrepancies in the data and that a 'perfect' separation was achieved between the unknown cases and the pharmaceutically manufactured tablets using this allocation rule on the training dataset. This meant that both models produced a sensitivity and specificity of 1 using both the training samples and the four test cases. The use of

the ROC Curve therefore produced the results that were closest to the original classification.

Table 8.6. Results of the ROC curve using data from the four test cases and the training samples.

		Model 1		Model 2	
		Unknown Origin	Pharmaceutical	Unknown Origin	Pharmaceutical
Method 3 ROC Curve	ROC Cut-Off Value	If D>0.42 classify as unknown otherwise pharmaceutical	If D<0.42 classify as pharmaceutical otherwise unknown	If D>0.75 classify as unknown otherwise pharmaceutical	If D<0.75 classify as pharmaceutical otherwise unknown
	Area Under Curve	1.00	1.00	1.00	1.00
Classification	Unknown Origin	2	0	2	0
	Pharmaceutical	0	2	0	2

8.5.5 Test of Assumptions and Suitability of Data

For the statistical technique to be successful, three assumptions are required for the use of LDA:

1. Independence and non-multiple linearity of variables
2. Multivariate normality
3. Homogeneity of variance/covariance matrix

Pearson correlation coefficient values greater than 0.3 increase the likelihood of false positives occurring, however, it has been argued that LDA can tolerate deviations from the assumption of multivariate normality and homogeneity of covariance (Brown and Wicker,2000).

8.5.5.1 Independence and non-multiple linearity.

The assumption of independence and non-multiple linearity can be demonstrated by a lack of correlation between the variables, with an ideal Pearson's correlation coefficient value of <0.3 between pairs on independent variables. Tests of the assumption of independence and non-collinearity for both models (Table 8.7) show that the correlation coefficient was below the recommended 0.3 level for PC1, 2 and 5 but the correlations to PC3 and 4 were slightly higher. Therefore, the LDA assumption of independence and non-collinearity is acceptable for the three discriminating independent variables in the model.

Table 8.7. Correlation between variables.

Model 1. Pooled Within Group Correlation Between Predictors

Correlation	PC1	PC2	PC3	PC4	PC5
PC1		-0.292	0.017	-0.595	-0.068
PC2	-0.292		-0.005	0.061	-0.103
PC3	0.017	-0.005		0.329	0.318
PC4	-0.595	0.061	0.329		-0.120
PC5	0.068	-0.103	0.319	-0.120	

Model 2. Pooled Within Group Correlation Between Predictors

Correlation	PC1	PC2	PC5
PC1		-0.292	0.068
PC2	-0.292		-0.103
PC5	0.068	-0.103	

8.5.5.2 Multivariate normality

The tests for multivariate normality are difficult to perform and are not generally included in most statistical packages but Brown and Wicker (2000) suggest that if the discriminant function scores are normally distributed then multivariate normality is likely. The Shapiro-Wilk test presents a null hypothesis that the population is normally distributed. The results of the Shapiro-Wilk test are shown in Table 8.8

and demonstrate that the data used for the LDA are normally distributed so this assumption of the LDA method is satisfied.

Table 8.8. Results of Shapiro Wilk Test of normality on values of D for each tablet group, where: W = the observed value of the Shapiro-Wilk statistic and p is the probability of the outcome. The number in brackets refers to the degrees of freedom. H0: Data are normally distributed. A p-value >0.05 indicates that the null hypothesis is true and the data are normally distributed. A p-value <0.05 results in the null hypothesis being rejected, indicating that the data are not normally distributed.

Model 1	Shapiro-Wilk	Null Hypothesis	Distribution
Unknown	W(8) = 0.996; p=1.000	Accepted	Normal
Pharmaceutical	W(8) = 0.825; p=0.053	Accepted	Normal
Model 2			
Unknown	W(8) = 0.909; p=0.348	Accepted	Normal
Pharmaceutical	W(8) = 0.880; p=0.190	Accepted	Normal

8.5.5.3 Homogeneity of variance/covariance matrix

The variance/covariance matrices should demonstrate similarity across groups and the assumption is examined by the Box's M test. The null hypothesis states that the covariance matrices do not differ and the significance level is set at p=0.01.

However, this is a very sensitive test and Garson (2012) suggested that the results of the Box's M test could be disregarded if the log determinants are similar. Some variation is expected but too much could denote the presence of outliers and inaccuracy of significance tests.

The population variation matrices were not equal, according to Box's M (Table 8.9). The log determinants are similar but not equal. Brown and Wicker suggest that violations of this assumption are permitted if the $n_{PHARMACEUTICAL}$ /$n_{UNKNOWN}$ <1.5 (Brown and Wicker,2000), which is true in this case.

Table 8.9. Results of Box's M Test and the log determinants, **where: F = the ratio of two variants (Degrees of Freedom 1, Degrees of Freedom 2) and p is the probability of the outcome.**

Model 1	Log Determinant	Box's M	Equation
Unknown	21.492	52.619 F(15,789) = 2.105; p<0.008	$\frac{n_{PHARMACEUTICAL}}{n_{UNKNOWN}}$ = 1.00
Pharmaceutical	18.062		
Model 2			
Unknown	16.144	19.689 F(6,1420) = 2.506; p<0.020	$\frac{n_{PHARMACEUTICAL}}{n_{UNKNOWN}}$ = 1.00
Pharmaceutical	13.760		

All of the assumptions were acceptable for this study.

8.5.6 Suitability of Data

An important step of the LDA was to ensure the data was suitable for this type of analysis. A discriminant procedure such as LDA requires variance between the independent variables in order to distinguish between groups. The Wilks' Lambda test evaluates the discriminating power of each of the independent variables. The test generates a Wilks' Lambda value which is the ratio between the within group variance and the total variance. Wilks' Lambda uses a scale of 0 – 1, where results of 0 indicate there is significant variation in the means of the groups, giving it total discriminatory power. Results of 1 signify that there is no difference in the groups' means and therefore no discriminatory potential. However, even if only one group variable is significant, then the whole model is regarded as significant (Garson, 2012).

The F test explores the ratio of variance and determines the level of significance. It stipulates that when $p<0.05$, the independent variable has a significant impact on discrimination. An F test with $p>0.05$ indicates that discrimination simply occurs by chance (Garson, 2012). In this study PC1, PC2 and PC5 have significant discriminatory power whereas, PC3 and PC4 have little discriminatory potential (Table 8.10). Wilks' Lambda can be used to refine a model by removal of the less discriminating functions (Garson, 2012). For this reason, two models were created for this project. Model 1 incorporated the statistical results produced by all five principal components and Model 2 was based on the results of only principal components 1, 2 and 5.

Table 8.10. Wilks' Lambda test for significance of independent variables.

	Wilks' Lambda	Discriminatory Significance
PC1	0.332	$F(1,14) = 28.15$; $p<0.00$
PC2	0.401	$F(1,14) = 20.88$; $p<0.00$
PC3	0.943	$F(1,14) = 0.85$; $p<0.373$
PC4	0.767	$F(1,14) = 4.25$; $p<0.058$
PC5	0.678	$F(1,14) = 6.66$; $p<0.022$

The aim of the discriminant analysis was to use the DSC data which represents the tablets' excipient content to separate the illicit cases into groups which originate from a pharmaceutical manufacturing process to those where the origin is unknown. Therefore, only one discriminant function, or allocation rule, was required to distinguish between the two groups. Wilks' Lambda was used to determine the effectiveness of the discriminant function. The null hypothesis stated that the group means are equal and therefore do not discriminate but a significant Wilks' Lambda score, as determined by both models in this project, indicates that the null hypothesis can be rejected (Garson, 2012). This means that both models were found to be discriminating (Table 8.11).

Table 8.11. Wilks' Lambda test of discriminant function significance and Eigenvalue results.

Model	Wilks' Lambda	Eigenvalue	% Variance	Canonical Correlation
Model 1	0.139 (p<0.00)	6.176	100	0.928
Model 2	0.154 (p<0.00)	5.491	100	0.920

Eigenvalues demonstrate the relative importance of each of the discriminant functions. For this project, only one discriminant function was required to classify the cases into two groups. Therefore, the eigenvalue should reflect 100% discrimination, as seen in Table 8.11.

The canonical correlation indicates that correlation was present between the group values and the values related to the discriminant function. As there was only one discriminant function, this value illustrates that most of the difference between the groups is determined by the one function and is therefore close to a value of 1 (Table 8.11) (Garson, 2012).

The standardized discriminant function coefficients (Table 8.12) reflect the relative importance of each of the independent variables (i.e. PCs 1 to 5) to the discriminant function and the classification results. When variables flagged as non-significant by the Wilks' Lambda test (Table 8.11) are removed (i.e. PC3 and PC4), the level of importance assigned to each of the remaining variables is affected (Model 2). The results of both models indicated that the greatest contribution was provided by PC1 (Table 8.12). This is expected since the first PC represents the largest portion of the variance in the original DSC dataset.

Table 8.12. Standardized discriminant function coefficients showing the relative importance of each principal component to the discriminant function. The results identify that the largest contribution for both models was given by PC1.

Model 1	PC1	PC2	PC3	PC4	PC5
Coefficients	1.073	0.817	-0.205	0.484	0.411
Model 2					
Coefficients	0.815	0.792			0.320

8.6 Conclusion

Illicit tablets can be distinguished from pharmaceutical tablets using a variety of analytical methods which examined either the nature and amount of active drug substance or the properties of the excipient. Two groups of illicit tablets contained an active drug substance other than diazepam (**1**) and another group contained quantities of diazepam (**1**) that were outside the specified level for a pharmaceutical tablet. This left a group of illicit tablets with the correct type and amount of active drug substance (10 mg diazepam **1**) but DSC analysis discriminated between these tablets and pharmaceutical tablets based on the tablets' excipient content. Linear discriminant analysis of the DSC data indicated that the excipient content of the unknown tablets was distinct from pharmaceutical diazepam (**1**) tablets licenced in the UK. However, this result does not rule out the possibility that the unknown tablets were pharmaceutically manufactured outside the UK and diverted for illicit use or that they had been pharmaceutically manufactured for the UK market and diverted but were older than the pharmaceutical tablets used in this study and had degraded over time. Degradation of the excipient could generate a DSC thermogram that was distinct from the newer tablets obtained directly from the pharmaceutical company. This possibility merits further investigation.

The LDA model had good predictive power as indicated by the LOOCV but one pharmaceutical and one unknown tablet in the training dataset were misclassified.

However, analysis of the four test cases and the training data using the centroid and confidence interval methods only misclassified one pharmaceutical tablet using the first model and two using the second. One possible reason for two of the pharmaceutical tablets being misclassified is that not all of the pharmaceutical tablets were run on the DSC on the same day. The first two cases (MA061 and MA064) were analysed along with the illicit cases. The remaining eight cases of pharmaceutical tablets were run later. It was these two pharmaceutical tablets (MA061 and MA064) which were misclassified, as they did not group with the remaining pharmaceutical tablets. The DSC instrument underwent maintenance during the interval between analyses and this may have had an effect on the outcome and explain why these two pharmaceutical tablets were misclassified by the model. Potentially, if the tablets had all been run together, it may have been more difficult to separate the pharmaceutical tablets from the unknown. This would be worth further investigation in the future and could help to produce a more effective model.

Despite this, the unknown tablets were correctly classified using the majority of methods. However, the LOOCV result achieved 87.5% accuracy, while the use of centroids and confidence intervals gave a result of over 90%. In addition the ROC Curve correctly identified all of the tablets. The limitation of this analysis was the small dataset but the results of this study indicate the strong potential in this statistical model.

Chapter 9. Conclusions and Recommendations for Future Work

9.1 Chapter Summary

The conclusion describes the context in which this study is set and provides a brief summary of the work performed and the results obtained.

This section also suggests ideas for future work to build upon the analysis performed during this project. Some investigations could have been performed in different ways and a variety of avenues could have been taken. Initial tests were also made into alternative methods of analysis such as DSC. Therefore, the work performed in this project has provided an important step on which to base future work and suggestions are described with a view to obtaining further information that would help in the characterisation of illicit tablets.

9.2 Conclusions

The results of this project have proved that differentiation can be made between tablets in the illegal supply chain by using information gained from their physical and chemical analysis. By characterising the illicit blue tablets and comparing the resulting data to results generated by similar analysis of known pharmaceutical tablets, differences emerged which enabled some distinction to be made between illicitly produced and pharmaceutically manufactured tablets. Comparison of the illicit tablets also revealed an array of different active drug substances in varying quantities in the illegal drug tablets. However, some similarities were also discovered between the illicit tablets, indicating possible connections between the seized cases, which may provide important intelligence to police forces.

Differences in tablet markings showed that many of the tablets in the illegal supply chain had not been pharmaceutically manufactured for the UK market, including tablets containing the letters EZ or NTZ. Some of the markings, such as MA / D10 were consistent with pharmaceutical logos that were no longer in production in the UK. However, due to shelf life, they may have been legally available at the time of

manufacture and then diverted for illegal use. Other cases contained tablets marked CP | D10, which correspond with the imprints used by Wockhardt and which are still being manufactured in the UK, indicating that they may also have been diverted into the illegal supply chain.

Weight analysis of the illicit tablets highlighted differences in the consistency of illicit cases. The weight range of pharmaceutical tablets was very small because of the requirement to comply with pharmaceutical regulations. Variation in tablet weight could ultimately affect the dosage level of the drug and is therefore carefully monitored within the pharmaceutical industry. However, greater variation of tablet weight existed within illicit cases. This provides an insight into illicit manufacturing procedures and can provide a quick and simple basis for initial screening of seized cases.

Similarly, tablets manufactured by for example, the pharmaceutical company MA Pharmachem, consistently weighed between 165 – 175 mg, however, twelve of the illicit cases with a corresponding MA | D/10 marking were found to have a mean weight above this level, therefore suggesting that these cases had been illicitly manufactured, or were possibly a failed pharmaceutical batch, which had not been destroyed. Another twelve illicit cases contained tablets that did correspond to the pharmaceutical range indicating they may have been pharmaceutically manufactured, but diverted tablets.

Greater variation was also found in tablet depth, within illicit cases compared to pharmaceutical batches and was emphasised through relative standard deviation. The data was analysed in conjunction with box plots and graphical representations which enabled differences to be clearly visualised.

Measurements of diameter, depth and weight allowed 14 cases of seized tablets to be identified as matching known pharmaceutical tablets. These 14 cases were comprised of ten cases marked MA and one case consistent with tablets produced by each of Tensium, Teva and Wockhardt. These companies have all been licensed to produce diazepam (**1**) tablets. The final case which matched the pharmaceutical tablets was marked Roche which is a legitimate manufacturer of diazepam (**1**)

tablets in other countries but has no license for the UK. Significantly, these 14 identified cases remained consistent with pharmaceutical tablets in all of the following chemical tests performed thus giving a viable indication that these cases had been pharmaceutically manufactured (albeit Roche was manufactured outside the UK) and had been diverted into the illegal supply chain.

The results of the physical analysis therefore demonstrated that difference in weight and depths of tablets can identify some cases which have been illicitly manufactured. Further analysis, such as through standard deviation and boxplots to explore the range of variation may also help to distinguish some cases which are of illicit origin. This new research into illicit street drugs seized in Scotland, has therefore identified a valuable initial step for future work in tablet discrimination.

Analysis by GC-MS revealed that only 38 of the 62 seized cases tested contained any diazepam (**1**). Instead, a variety of active ingredients were present in the illicit tablets, including some such as etizolam (**7**) and phenazepam (**16**), which are not licensed for use in the UK. This important technique was therefore able to identify many tablets which had not been pharmaceutically manufactured for the UK market. As no work has previously investigated the active ingredient present in the illicit blue tablets seized in Scotland, GC-MS revealed how vital this is, in order to provide up to date information for both police and medical services.

Work performed on the GC-MS also highlighted the growing sophistication of illicit manufacturing processes, with lubricants being detected in illicit tablets. This indicates an increase in knowledge and availability of equipment.

Although not a confirmatory technique, comparison of analyte retention times by HPLC was able to support the GC-MS identifications while also providing information regarding the quantity of diazepam (**1**) present in the tablets. HPLC revealed there was a variation from around 8 – 48 mg in the amount of active drug substance present in the diazepam (**1**) tablets. Only 20 of the 38 cases identified with any diazepam (**1**) by GC-MS, were found to contain the correct amount of diazepam (**1**) (around 9 - 11 mg). Two of the cases were discovered to contain a slightly lower amount (around 8 mg) and 17 cases contained substantially higher than the

expected quantity (approximately 19 – 48 mg). The remaining tablets contained other active drug substances. HPLC analysis therefore revealed that many of the tablets containing diazepam (**1**) on the illicit market, had not been pharmaceutically manufactured and reflects a potential change away from diverting pharmaceutical tablets to illicit manufacture.

This vital investigation was therefore able to distinguish between cases containing different levels of active drug substance and revealed the true nature of diazepam (**1**) being sold on the streets of Tayside. This again may provide useful information to both police and medical staff.

Use of DSC proved very informative and provided a thermal profile which was largely based on the excipients present in the analysed tablets, due to the small amount of active drug substance present in diazepam (**1**) tablets. DSC thermograms clearly showed differences and similarities between the illicit cases and in comparison to the pharmaceutical tablets. This therefore proved valuable for distinguishing between the various cases and for revealing potential links which may have otherwise been missed. Although unable to identify excipients, the technique gave an indication of substances present, such as lactose, which gives a distinctive trace and is often a major component of both pharmaceutical and some illicit tablets. The use of DSC proved invaluable for this project and has demonstrated great potential for future projects.

The DSC thermograms also provided data influenced by the excipients, which was subjected to statistical cluster analysis using k-means clustering. Data reduction using principal component analysis was also performed on the data and allowed linear discriminant analysis to be performed on the seized cases which were most similar to the pharmaceutical tablets. By using three different allocation rules, the results indicated that illicit tablets were able to be distinguished from the pharmaceutical tablets. This novel investigation into illicit tablets using a combination of DSC and statistical analysis has demonstrated how powerful the technique can be, by exploring finer detail of the main constituents of the tablets and distinguishing all cases from the pharmaceutical tablets. This new and innovative

technique, developed from well-established methodologies could provide vital insights in future work.

Statistical cluster analysis using AHC, k-means clustering and a subjective approach based on visual comparison, was performed using three sets of independent data generated by each of the analytical techniques. The results of the analyses were combined in a heat map which, highlighted how similar different illicit cases were to each other and to analysed pharmaceutical tablets based on statistical analysis of their physical and chemical properties. The heat map identified seized cases which clustered with the pharmaceutical batches a varying number of times, which could suggest that they had been pharmaceutically manufactured and diverted into the illegal supply chain. In addition, a number of the illicit cases were found to cluster together on multiple occasions, implying there may be a link between the seized cases. The heat map proved to be a useful and informative way of combining results from multiple tests and an effective method of visualising cases which shared certain characteristics.

Study into illicit diazepam (**1**) tablets had not previously been performed and this work has successfully provided a greater understanding of “blue tablets” (allegedly diazepam **1**) in the Scottish illicit market. In addition, an investigation into the forensic use of DSC has demonstrated the potential value that thermal analysis could provide in future investigations. The study has also shown how physical and chemical characterisation and statistical analysis can successfully be combined to both differentiate between tablets and to highlight potential links. The results have indicated that of the twenty-four MA / D10 marked tablets (which were used as the sample set) obtained from the Scottish Police Authority there is a high probability that five are diverted pharmaceutical tablets, with another five potential diverted tablets. The remaining fourteen are illicit.

9.3 Recommendations for Future Work

Based on the work performed a number of recommendations for future work can be suggested to build on the knowledge already gained. Firstly, the great potential offered by DSC would require a substantial amount of further investigation. Reruns

of tablets suggest that changes take place over time and investigation into effects of time and storage conditions would provide greater understanding of the limitations of the technique. This could reveal that direct comparison of different cases is difficult as resulting thermograms may show variation despite tablets originating from the same source. However, it may also provide an understanding of how each excipient would behave over time thus helping to identify links been illicit seizures. Consequently, the forensic analysis of tablets using DSC would benefit from further research in this area.

Some differences between samples may also be due to inconsistent blending of the tablet and the small sample size. Therefore, it may be advisable to analyse more samples from each tablet and case. It should be noted however, that samples that were used in this project were those obtained by Police Scotland so the sample size was dependent on the seizures.

The sensitivity of DSC had shown that tablets could be separated according to visual comparison of the thermograms produced. The tablets marked MA / D10 for example, could be separated into groups similar to those distinguished by visual comparison (Chapter 7 – Statistical Cluster Analysis). The difference being the amalgamation of groups 1 and 2 through comparison of thermograms. However, this was not due to the inability of DSC but due to the subjective nature of deciding a cut-off point by which to distinguish between levels of diazepam (**1**) detected. Interestingly, analysis of spectra produced testing the use of near infrared spectroscopy (NIR) for this project, had only been able to separate the cases into three groups. Groups 1 and 2 with varying amounts of diazepam (**1**) again looked similar but the cases containing etizolam (**7**) also appeared comparable making it difficult to distinguish between the spectra produced by cases containing diazepam (**1**) and etizolam (**7**). This would suggest that DSC may be a more sensitive technique for visual comparison of traces produced. This would be supported by the study by Matos *et al*. (2017) whose compatibility studies using both DSC and infrared spectroscopy found that interaction between diazepam (**1**) and colloidal silicon dioxide produce a lower enthalpy value, which was not detected by infrared spectroscopy alone, suggesting that DSC is a more sensitive technique. However, a

more detailed investigation into the benefits and comparison of each technique may be required to assess whether the two methods could complement each other.

DSC thermograms show initial moisture loss from tablets through the presence of a gentle endothermic slope from the start of the trace. It is possible that this may hide events affecting the lower level constituents and could also affect data used for statistical analysis depending on the amount present. Therefore, for this project, the temperature range up to 100 ^{o}C was disregarded in the statistical analysis. Future work could benefit from pre-drying tablets for DSC analysis to assess whether further information could be revealed.

Inclusion of Thermal Gravimetric Analysis would also be interesting to compare to DSC analysis, as it would help to clarify thermal events taking place in the sample. In tests performed as part of this research, TGA was able to clarify instances of dehydration or melting because of differences in weight loss. This could be valuable particularly if endotherms from melting were obscured by a broader endotherm produced by dehydration.

Other work that could prove useful would be colour analysis and granularity of tablets. Identifying a means of describing colour that is not subjective and allows for mottled tablets, would help with the classification. Uneven colour could be to do with an uneven blend but variance in the level of dye added between different batches, may also change perception of the colour. However, analysis of dyes may indicate that the same food dyes are used, which would not help to distinguish between cases. The use of photography may be a more realist approach and may also be combined with assessing granularity. The granularity of tablets gives an indication of the methods of production used and could provide useful intelligence for the police.

Identification of sugars could determine whether the same excipients were used by the pharmaceutical companies and those produced illegally and could highlight differences or similarities between different illicit seizures. Similarly, although investigations into tablet markings and damage analysis could prove problematic, largely due to tableting machines with multiple stations, overlaying photographs of similarly marked cases may again help to identify links between seizures.

By including information related to where each seizure was made could also prove interesting because when combined with information relating to potential links between cases, this could indicate how widespread the market is for a particular illicit manufacturer.

This work has a number of avenues which could be pursued and a potential first step would be to approach the procurator fiscal to increase the number of tablets analysed, thus giving a greater understanding of the illicit “blue tablet” market. Over the time this project has been performed, differences have been witnessed in the types of tablets received. Many of the first batch of seizures received from the police contained tablets with 10 mg of diazepam (**1**) and only two cases which contained another active drug substance (phenazepam **16**). By the time the last batch was received, six of the twenty-two cases contained diazepam (**1**) and, of those, only two contained the pharmaceutical amount of between 9 – 11 mg. This would indicate that more illegally manufactured tablets were entering the illicit market than pharmaceutically diverted tablets. The market is constantly changing, possibly due to using whichever active drug substance is the most easily accessible and the availability of tableting equipment. This highlights the importance of the work started in this project and the need to continue with the work in order to provide police and medical staff with up to date information on what is being bought and sold on the streets of Scotland.

The final overall aim of this and later projects would be to develop a rapid robust system for determining the active ingredients in seized drug tablets and, along with some of the techniques described herein, be able to provide highly useful drug intelligence for police forces.

Publications Arising from this Work

A preliminary investigation to group disparate batches of licit and illicit diazepam tablets using differential scanning calorimetry.

Authors:

S. Bibi,[a] D. H. Bremner,[b] M. Macdougall-Heasman,[b] R. Reid,[a] K. Simpson,[c] A. Tough,[a] S. Waddell,[a] I. J. Stewart[b] and K. H. Matthews*[a]

a: Robert Gordon University, Aberdeen
b: Abertay University, Dundee

Published in:

Analytical Methods, 20 (7), pp. 8597-8604.

Appendices

Appendix I - Statistical Techniques Applied

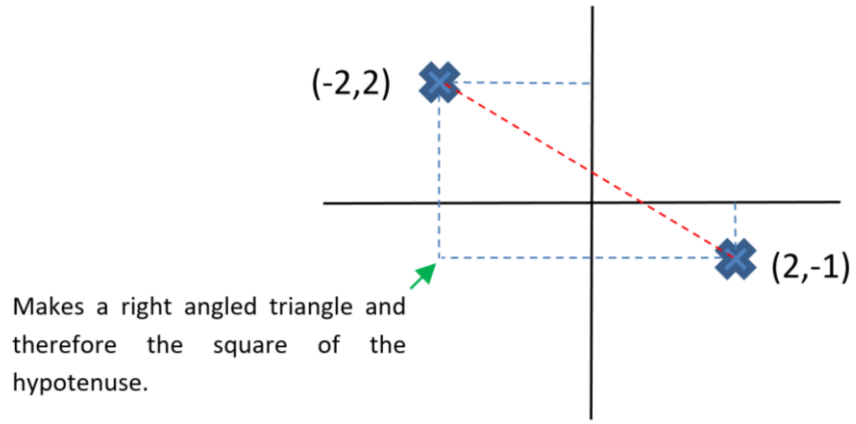
1. Euclidean Distance
2. Agglomerative Hierarchical Clustering
3. K-Means Clustering
4. Principal Component Analysis
5. Linear Discriminant Analysis
6. ‘R’ Scripts used for PCA Analysis

Each technique is demonstrated using the data produced by chemical and physical analysis in this project, where possible.

Euclidean Distance

$$\sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$$

It is the distance of a straight line between two points.



The source of this formula is the Pythagorean theorem,

ie. The $(\text{hypotenuse})^2 = 3^2 + 4^2 = 9 + 16 = 25$

$\sqrt{25} = 5$

Therefore Euclidean Distance between points (2,-1) and (-2,2) =

Dist ((2,-1), (-2,2)) = $\sqrt{(2 - (-2))^2 + ((-1) - 2)^2}$

$= \sqrt{(2+2)^2 + (-1-2)^2}$

$= \sqrt{(4)^2 + (-3)^2}$

$= \sqrt{16 + 9}$

$= \sqrt{25}$

$= 5$

Therefore using data relating to cases 26, 31, 13, 83, 16, 74, indicating their diazepam (**1**) levels and relative standard deviation of weight of four tablets taken from each case, a distance matrix showing the distance between each of the points is required.

In order to determine the matrix, the distance between each of the points needs to be calculated.

Cases 26 – 31

Points (25, 3.06) and (22, 1.99) =

Dist ((25, 3.06), (22, 1.99)) = $(25 -22)^2 + (3.06 – 1.99)^2$

$= \sqrt{(25 - 22)^2 + (3.06 – 1.99)^2}$

$= \sqrt{(3)^2 + (-1.07)^2}$

$= \sqrt{9 + 1.1449}$

$= \sqrt{10.1449}$

$= 3.185$

Cases 26 – 13

Points (25, 3.06) and (22, 1.99) =

Dist ((25, 3.06), (22, 1.27)) = $(25 -22)^2 + (3.06 – 1.27)^2$

$= \sqrt{(25 - 22)^2 + (3.06 – 1.27)^2}$

$= \sqrt{(3)^2 + (-1.79)^2}$

$= \sqrt{9 + 3.204}$

$= \sqrt{12.204}$

$= 3.493$

Cases 26 – 83

Points (25, 3.06) and (9, 0.92) =

Dist ((25, 3.06), (9, 0.92)) = $(25 - 9)^2 + (3.06 – 0.92)^2$

$= \sqrt{(25 - 9)^2 + (3.06 – 0.92)^2}$

$= \sqrt{(16)^2 + (3.98)^2}$

$= \sqrt{256 + 15.8404}$

$= \sqrt{271.8404}$

$= 16.488$

Cases 26 – 16

Points (25, 3.06) and (10, 0.82) =

Dist ((25, 3.06), (10, 0.82)) = $(25 -10)^2 + (3.06 – 0.82)^2$

$= \sqrt{(25 - 10)^2 + (3.06 – 0.82)^2}$

$= \sqrt{(15)^2 + (2.24)^2}$

$= \sqrt{225 + 5.0176}$

$= \sqrt{230.0176}$

$= 15.166$

Cases 26 – 74

Points (25, 3.06) and (9, 1.32) =

Dist ((25, 3.06), (9, 1.32))

$$= \sqrt{(25 - 9)^2 + (3.06 - 1.32)^2}$$

$$= \sqrt{(16)^2 + (1.74)^2}$$

$$= \sqrt{256 + 3.0276}$$

$$= \sqrt{259.0276}$$

$$= 16.094$$

Cases 31 – 13

Points (22, 1.99) and (22, 1.27) =

Dist ((22, 1.99), (22, 1.27)) =

$$= \sqrt{(22 - 22)^2 + (1.99 - 1.27)^2}$$

$$= \sqrt{(0)^2 + (0.72)^2}$$

$$= \sqrt{0 + 0.5184}$$

$$= 0.72$$

Cases 31 – 83

Points (22, 1.99) and (9, 0.92) =

Dist ((22, 1.99), (9, 0.92)) =

$$= \sqrt{(22 - 9)^2 + (1.99 - 0.92)^2}$$

$$= \sqrt{(13)^2 + (2.91)^2}$$

$$= \sqrt{169 + 8.4681}$$

$$= \sqrt{177.4681}$$

$$= 13.222$$

Cases 31 – 16

Points (22, 1.99) and (10, 0.82) =

Dist ((22, 1.99), (10, 0.82)) =

$$= \sqrt{(22 - 10)^2 + (1.99 - 0.82)^2}$$

$$= \sqrt{(12)^2 + (1.17)^2}$$

$$= \sqrt{144 + 1.3689}$$

$$= \sqrt{145.3689}$$

$$= 12.057$$

Cases 31 – 74

Points (22, 1.99) and (9, 1.32) =

Dist ((22, 1.99), (9, 1.32)) =

$$= \sqrt{(22 - 9)^2 + (1.99 - 1.32)^2}$$

$$= \sqrt{(13)^2 + (0.67)^2}$$

$$= \sqrt{169 + 0.4489}$$

$$= \sqrt{169.4489}$$

$$= 13.017$$

Cases 13 – 83

Points (22, 1.27) and (9, 0.92) =

Dist ((22, 1.27), (9, 0.92)) =

$$= \sqrt{(22 - 9)^2 + (1.27 - 0.92)^2}$$

$$= \sqrt{(13)^2 + (0.35)^2}$$

$$= \sqrt{169 + 0.1225}$$

$$= \sqrt{169.1225}$$

$$= 13.005$$

Cases 13 – 16

Points (22, 1.27) and (10, 0.82) =

Dist ((22, 1.27), (10, 0.82)) =

$$= \sqrt{(22 - 10)^2 + (1.27 - 0.82)^2}$$

$$= \sqrt{(12)^2 + (0.45)^2}$$

$$= \sqrt{144 + 0.2025}$$

$$= \sqrt{144.2025}$$

$$= 12.008$$

Cases 13 – 74

Points (22, 1.27) and (9, 1.32) =

Dist ((22, 1.27), (9, 1.32)) =

$$= \sqrt{(22 - 9)^2 + (1.27 - 1.32)^2}$$

$$= \sqrt{(13)^2 + (-0.05)^2}$$

$$= \sqrt{169 + 0.0025}$$

$$= \sqrt{169.0025}$$

$$= 13.00$$

Cases 83 – 16

Points (10, 0.82) and (9, 0.92) =

Dist ((10, 0.82), (9, 0.92)) =

$$= \sqrt{(10 - 9)^2 + (0.82 - 0.92)^2}$$

$$= \sqrt{(1)^2 + (-0.1)^2}$$

$$= \sqrt{1 + 0.01}$$

$$= \sqrt{1.01}$$

$$= 1.005$$

Cases 83 - 74

Points (9, 1.32) and (9, 0.92) =

Dist ((9, 1.32), (9, 0.92)) =

$$= \sqrt{(9 - 9)^2 + (1.32 - 0.92)^2}$$

$$= \sqrt{(0)^2 + (0.4)^2}$$

$$= \sqrt{0 + 0.16}$$

$$= \sqrt{0.16}$$

$$= 0.4$$

Cases 16 – 74

Points (10, 0.82) and (9, 1.32) =

Dist ((10, 0.82), (9, 1.32)) =

$$= \sqrt{(10 - 9)^2 + (0.82 - 1.32)^2}$$

$$= \sqrt{(1)^2 + (-0.5)^2}$$

$$= \sqrt{1 + 0.25}$$

$$= \sqrt{1.25}$$

$$= 1.118$$

Distance Matrix

Table 10.3.1 Distance matrix produced using data from six seized cases.

Distance	Case 13	Case 16	Case 26	Case 31	Case 74	Case 83
Case 13	-	12.008	3.493	0.72	13.00	13.005
Case 16	12.008	-	15.166	12.057	1.118	1.005
Case 26	3.493	15.166	-	3.185	16.094	16.488
Case 31	0.72	12.057	3.185	-	13.017	13.222
Case 74	13.00	1.118	16.094	13.017	-	0.400
Case 83	13.005	1.005	16.488	13.222	0.400	-

Agglomerative Hierarchical Clustering

Agglomerative Hierarchical Clustering (AHC) uses the Euclidean distances demonstrated in section 10.3.1 - Euclidean Distance above.

Table 10.3.2 Distance matrix showing Euclidean distance between 6 seized cases.

Distance	Case 13	Case 16	Case 26	Case 31	Case 74	Case 83
Case 13	-	12.008	3.493	0.72	13.00	13.005
Case 16	12.008	-	15.166	12.057	1.118	1.005
Case 26	3.493	15.166	-	3.185	16.094	16.488
Case 31	0.72	12.057	3.185	-	13.017	13.222
Case 74	13.00	1.118	16.094	13.017	-	0.400
Case 83	13.005	1.005	16.488	13.222	0.400	-

In each iteration, the closest pairs in the distance matrix (Table 10.3.2) are grouped into one cluster.

Step 1.

Using the distances calculated for the cases and shown in the tablet, the closest pairs in the distance matrix are cases 74 and 83 at a distance of 0.4.

The distance matrix is then recalculated (Table 10.3.3), using the single linkage rule, where the minimum distance is specified so that:

d (74,83) -> 13 = min (^{d}74-13, ^{d}83-13) = min (13.00, 13.005) = 13.00

d (74,83) -> 16 = min (^{d}74-16, ^{d}83-16) = min (1.118, 1.005) = 1.005

d (74,83) -> 26 = min (^{d}74-26, ^{d}83-26) = min (16.094, 16.488) = 16.094

d (74,83) -> 31 = min (^{d}74-31, ^{d}83-31) = min (13.017, 13.222) = 13.017

Table 10.3.3 Recalculated distance matrix where cases 74 and 83 are combined.

Distance	Case 13	Case 16	Case 26	Case 31	(Cases 74,83)
Case 13	-	12.008	3.493	0.72	13.00
Case 16	12.008	-	15.166	12.057	1.005
Case 26	3.493	15.166	-	3.185	16.094
Case 31	0.72	12.057	3.185	-	13.017
Cases (74,83)	13.00	1.005	16.094	13.017	-

Step 2.

The step is repeated with the next closest clusters. In this example, the next closest are cases 13 and 31 at a distance of 0.72.

The distance matrix is recalculated as follows:

d (13,31) -> 16 = min (^{d}13-16, ^{d}31-16) = min (12.008, 12.057) = 12.008

d (13,31) -> 26 = min (^{d}13-26, ^{d}31-26) = min (3.493, 3.185) = 3.185

d (13,31) -> (74,83) = min (^{d}13-74, ^{d}13-83m ^{d}31-74, ^{d}31-83), using original distance matrix.

Therefore = min (13.00, 13.005, 13.017, 13.222) = 13.00

Table 10.3.4 Recalculated distance matrix where cases 74 and 83; and cases 13 and 31 are combined.

Distance	Cases (13,31)	Case 16	Case 26	Cases (74,83)
Cases (13,31)	-	12.008	3.185	13.00
Case 16	12.008	-	15.166	1.005
Case 26	3.185	15.166	-	16.094
Cases (74,83)	13.00	1.005	16.094	-

Step 3.

The step is repeated with the next closest clusters. In this example, the next closest are clusters (64, 83) and case 16 at a distance of 1.005.

The distance matrix is recalculated as follows:

d ((74,83),16) -> (13,31) = min (^{d}74-13, ^{d}74-31, ^{d}83-13, ^{d}83-31, ^{d}16-13, ^{d}16-31)

= min (13.00, 13.017, 13.005, 13.222, 12.008, 12.057) = 12.008

d ((74,83),16) -> 26 = min (^{d}74-26, ^{d}83-26, ^{d}16-26)

= min (16.094, 16.488, 15.166) = 15.166

Table 10.3.5 Recalculated distance matrix where cases 16, 74 and 83; and cases 13 and 31 are combined.

Distance	**Cases (13,31)**	**Case 26**	**Cases ((74,83)16)**
Cases (13,31)	-	3.185	12.008
Case 26	3.185	-	15.166
Cases ((74,83)16)	12.008	15.166	-

Step 4.

The next closest are clusters (13, 31) and case 26 at a distance of 3.185.

The distance matrix is recalculated as follows:

d ((13,31),26) -> ((74,83),16)

= min (^{d}13-74, ^{d}13-83, ^{d}13-16, ^{d}31-74, ^{d}31-83, ^{d}31-16, ^{d}26-74, ^{d}26-83, ^{d}26-16)

= min (13.00, 13.005, 12.008, 13.017, 13.222, 12.057, 16.094, 16.488, 15.166)

= 12.008

Table 10.3.6 Recalculated distance matrix where cases 16, 74 and 83; and cases 13, 26 and 31 are combined.

Distance	Cases ((13,31)26)	Cases ((74,83)16)
Cases ((13,31)26)	-	12.008
Cases ((74,83)16)	12.008	-

Step 5.

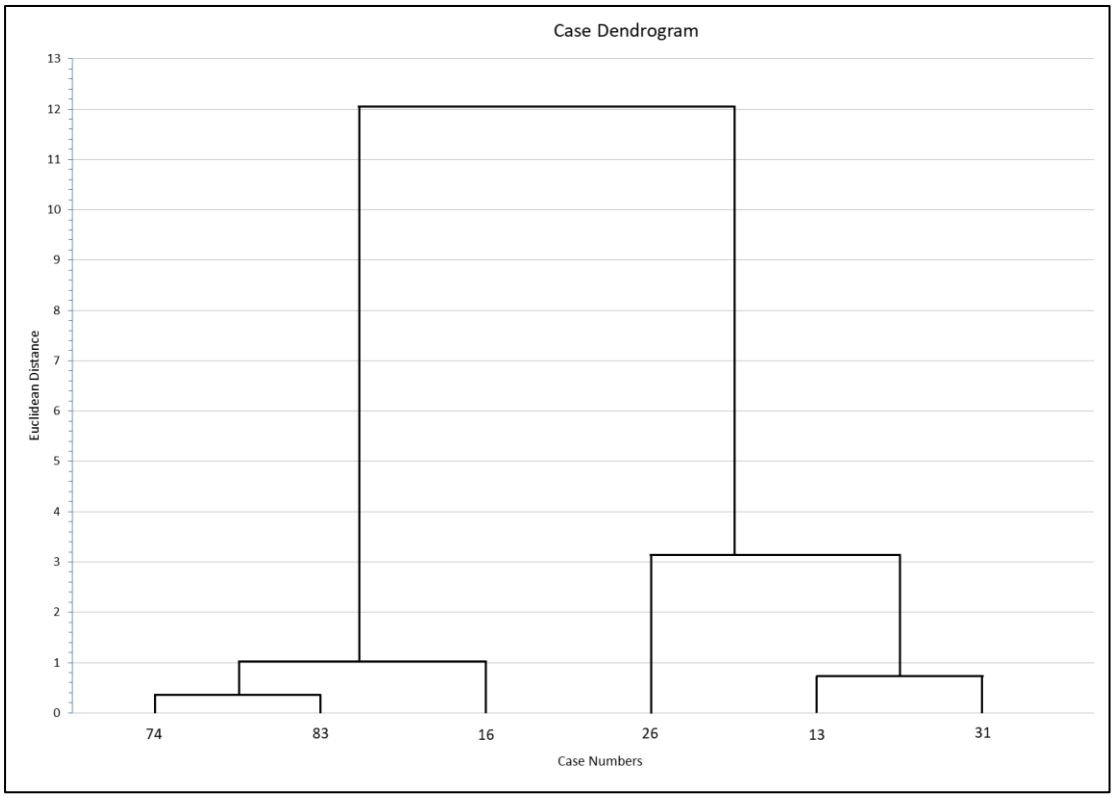
The remaining two clusters are merged.

Results

1. Six original clusters
2. Merge 74 and 83 at 0.4
3. Merge 13 and 31 at 0.72
4. Merge (74, 83) and 16 at 1.005
5. Merge (13, 31) and 26 at 3.185
6. Merge ((74, 83),16) and ((13, 31), 26 at 12.008

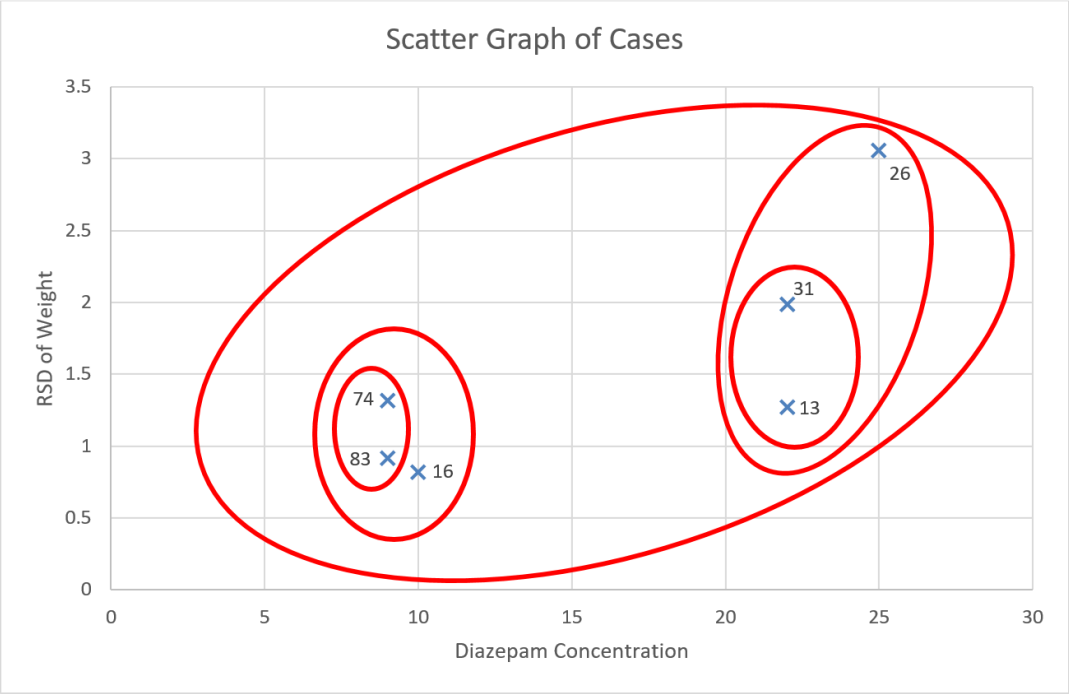
The results can be drawn on a dendrogram (Figure 10.3.1).

Figure 10.3.1 Dendrogram of the Agglomerative Hierarchical Clustering Results.



By returning to the original data points produced by analysis of diazepam (**1**) content and relative standard deviation of weight, a scatter graph can be plotted in x, y space and the hierarchy shown (Figure 10.3.2).

Figure 10.3.2 Scatter graph showing the hierarchy of the clustering Results.



K-Means Clustering

Data related to the diazepam (**1**) concentration and relative standard deviation of weight produced from four tablets taken from each of the seized cases 26, 31, 13, 83, 16, 74 is used to demonstrate k-means clustering. The data is shown in table 10.3.7.

Table 10.3.7 Diazepam (1) concentration and relative standard deviation recorded for six seized cases.

Case Number	Diazepam Concentration (mg)	Relative Standard Deviation of Weight
13	22	1.27
16	10	0.82
26	25	3.06
31	22	1.99
74	9	1.32
83	9	0.92

K-Means clustering uses two points which are furthest away from each other according to Euclidean distance, to become central vectors for two clusters.

Step 1.

The two furthest away points in the demonstration data are cases 26 and 83, using Euclidean Distance. These become the mean vectors for two clusters.

Table 10.3.8 Initial centroids created using Euclidean distance based on data produced by six seized cases.

Cluster	Case	Centroid (Mean Vector)
1	26	25, 3.06
2	83	9, 0.92

Step 2.

Working through each of the remaining cases, each are assigned to the nearest centroid and the mean is recalculated. So:

Case 13:

	Cluster	Case	Centroid (Mean Vector)
	1	26, 13	23.5, 2.165
Unchanged	2	83	9, 0.92

Case 16:

	Cluster	Case	Centroid (Mean Vector)
Unchanged	1	26, 13	23.5, 2.165
	2	83, 16	9.5, 0.87

Case 31:

	Cluster	Case	Centroid (Mean Vector)
	1	26, 13, 31	23, 2.107
Unchanged	2	83, 16	9.5, 0.87

Case 74:

	Cluster	Case	Centroid (Mean Vector)
Unchanged	1	26, 13, 31	23, 2.107
	2	83, 16, 74	9.333, 1.02

Step 3.

To ensure correct clustering, each subject is compared to both cluster means.

Case 13 -> Centroid of group 1

Points (23, 2.107) and (22, 1.27) =

Dist ((23, 2.107) (22, 1.27)) =

$$= \sqrt{(23 - 22)^2 + (2.107 - 1.27)^2}$$

$$= \sqrt{(1)^2 + (0.837)^2}$$

$$= \sqrt{1 + 0.7006}$$

$$= \sqrt{1.706}$$

$$= 1.306$$

Case 13 -> Centroid of group 2

Points (22, 1.27) and (9.333, 1.02) =

Dist ((22, 1.27) (9.333, 1.02)) =

$$= \sqrt{(22-9.333)^2 + (1.27-1.02)^2}$$

$$= \sqrt{(12.667)^2 + (0.25)^2}$$

$$= \sqrt{160.453 + 0.0625}$$

$$= \sqrt{160.5155}$$

$$= 12.670$$

Case 16 -> Centroid of group 1

Points (10, 0.82) and (23, 2.107) =

Dist ((10, 0.82) (23, 2.107)) =

$$= \sqrt{(10-23)^2 + (0.82-2.107)^2}$$

$$= \sqrt{(-13)^2 + (1.287)^2}$$

$$= \sqrt{169 + 1.656}$$

$$= \sqrt{170.656}$$

$$= 13.064$$

Case 16 -> Centroid of group 2

Points (10, 0.82) and (9.333, 1.02) =

Dist. ((10, 0.82) (9.333, 1.02)) =

$$= \sqrt{(10-9.333)^2 + (0.82-1.02)^2}$$

$$= \sqrt{(0.667)^2 + (-0.20)^2}$$

$$= \sqrt{0.445 + 0.04}$$

$$= \sqrt{0.485}$$

$$= 0.696$$

Case 26 -> Centroid of group 1

Points (25, 3.06) (23, 2.107) =

Dist. ((25, 3.06) (23, 2.107)) =

$$= \sqrt{(25 - 23)^2 + (3.06 – 2.107)^2}$$

$$= \sqrt{(2)^2 + (0.953)^2}$$

$= \sqrt{4 + 0.908}$

$= \sqrt{4.908}$

$= 2.215$

Case 26-> Centroid of group 2

Points (25, 3.06) and (9.333, 1.02) =

Dist. ((25, 3.06) (9.333, 1.02)) =

$= \sqrt{(25 - 9.333)^2 + (3.06 - 1.02)^2}$

$= \sqrt{(15.667)^2 + (2.04)^2}$

$= \sqrt{245.455 + 4.162}$

$= \sqrt{249.617}$

$= 15.799$

Case 31 -> Centroid of group 1

Points (22, 1.99) and (23, 2.107) =

Dist. ((22, 1.99) (23, 2.107)) =

$= \sqrt{(22 - 23)^2 + (1.99 - 2.107)^2}$

$= \sqrt{(1)^2 + (0.117)^2}$

$= \sqrt{1 + 0.0137}$

$= \sqrt{1.014}$

$= 1.007$

Case 31 -> Centroid of group 2

Points (22, 1.99) and (9.333, 1.02) =

Dist. ((22, 1.99) (9.333, 1.02)) =

$$= \sqrt{(22 - 9.333)^2 + (1.99 - 1.02)^2}$$

$$= \sqrt{(12.667)^2 + (0.97)^2}$$

$$= \sqrt{160.453 + 0.941}$$

$$= \sqrt{161.394}$$

$$= 12.704$$

Case 74 -> Centroid of group 1

Points (9, 1.32) and (23, 2.107) =

Dist. ((9, 1.32) (23, 2.107)) =

$$= \sqrt{(9 - 23)^2 + (1.32 - 2.107)^2}$$

$$= \sqrt{(-14)^2 + (-0.787)^2}$$

$$= \sqrt{196 + 0.619}$$

$$= \sqrt{196.619}$$

$$= 14.022$$

Case 74 -> Centroid of group 2

Points (9, 1.32) and (9.333, 1.02) =

Dist. ((9, 1.32) (9.333, 1.02)) =

$$= \sqrt{(9 - 9.333)^2 + (1.32 - 1.02)^2}$$

$$= \sqrt{(-0.333)^2 + (0.3)^2}$$

$$= \sqrt{0.111 + 0.09}$$

$$= \sqrt{0.201}$$

$$= 0.448$$

Case 83 -> Centroid of group 1

Points (9, 0.92) and (23, 2.107) =

Dist. ((9, 0.92) (23, 2.107)) =

$$= \sqrt{(9 - 23)^2 + (0.92 - 2.107)^2}$$

$$= \sqrt{(-14)^2 + (-1.187)^2}$$

$$= \sqrt{196 + 1.409}$$

$$= \sqrt{197.409}$$

$$= 14.050$$

Case 83 -> Centroid of group 2

Points (9, 0.92) and (9.333, 1.02) =

Dist. ((9, 0.92) (9.333, 1.02)) =

$$= \sqrt{(9 - 9.333)^2 + (0.92 - 1.02)^2}$$

$$= \sqrt{(-0.333)^2 + (-0.1)^2}$$

$$= \sqrt{0.111 + 0.01}$$

$$= \sqrt{0.121}$$

$$= 0.348$$

Results

The results of the k-means clustering are shown in Table 10.3.3.

Table 10.3.3 Results of k-means clustering of six seized cases.

Case	Distance to centroid of Cluster 1	Distance to centroid of Cluster 2
13	1.306	12.670
16	13.064	0.696
26	2.215	15.799
31	1.007	12.704
74	14.022	0.448
83	14.050	0.348

The results of the k-means clustering indicates that all cases remain in the same clusters and no further iterations are required. The final clusters created are shown in Table 10.3.4.

Table 10.3.9 Final clustering results determined by k-means clustering of six seized cases.

Cluster	Case	Centroid (Mean Vector)
1	26, 13, 31	23, 2.107
2	83, 16, 74	9.333, 1.02

Principal Component Analysis

To work out PCA on data, the first step is to zero the mean of the variables. In order to do this, the mean has to be calculated for each row of variables, then it needs to be subtracted from each of the variables to zero them. The equation to find the mean is:

$$\vec{\mu} = \frac{1}{n}(\vec{x}_1 + ... + \vec{x}_n)$$

Where $\vec{\mu}$ is the mean in vector form, n is the number of samples $\vec{x}$ and represents the samples in vector form.

Therefore matrix A becomes:

$$\begin{bmatrix} a_1-\mu_1 & b_1-\mu_1 & c_1-\mu_1 \\ a_2-\mu_2 & b_2-\mu_2 & c_1-\mu_2 \\ a_3-\mu_3 & b_3-\mu_3 & c_3-\mu_3 \\ \downarrow & \downarrow & \\ a_n-\mu_n & b_n-\mu_n & c_n-\mu_n \end{bmatrix}$$

Using data produced by Cases 2, 3 and 4, using the differential scanning calorimeter for the first three blocks of temperature, with data rounded to two decimal places:

Temperature	Case 2	Case 3	Case 4
100 – 105 °C	-1082.37	-1111.79	-882.15
105 – 110 °C	-1107.89	-1176.85	-902.51
110 – 115 °C	-1133.25	-1250.24	-927.76

The mean of each row was calculated as: 100 – 105 °C = 1025.44

105 – 110 °C = 1062.42

110 – 105 °C = 1103.75

So matrix A is:

$$\begin{bmatrix} -1082.37 - (-1025.44) & -1111.79 - (-1025.44) & -882.15 - (-1025.44) \\ -1107.89 - (-1062.42) & -1176.85 - (-1062.42) & -902.51 - (-1062.42) \\ -1133.25 - (-1103.75) & -1250.24 - (-1103.75) & -927.76 - (-1103.75) \end{bmatrix}$$

$$= \begin{bmatrix} -56.93 & -86.35 & 143.29 \\ -45.47 & -114.43 & 159.91 \\ -29.50 & -146.49 & 176.00 \end{bmatrix}$$

A covariance matrix (S) is then created to provide for relationship between variables. It is determined by using the equation:

$$S = \frac{1}{n-1} AA^T$$

Where A^T is the transpose of matrix A.

For our example, *n*=3 therefore $\frac{1}{n-1} = \frac{1}{2}$

So, $S = \frac{1}{2} AA^T$

$$= B \begin{bmatrix} -56.93 & -86.35 & 143.29 \\ -45.47 & -114.43 & 159.91 \\ -29.50 & -146.49 & 176.00 \end{bmatrix} \quad B^T \quad x \quad \begin{bmatrix} -56.93 & -45.47 & -29.50 \\ -86.35 & -114.43 & -146.49 \\ 143.29 & 159.91 & -176.00 \end{bmatrix}$$

$$= \begin{bmatrix} 31229.37 & 35383.14 & 39547.89 \\ 35383.14 & 40732.95 & 46248.38 \\ 39547.89 & 46248.38 & 53305.57 \end{bmatrix} \times \frac{1}{2}$$

$$S = \begin{bmatrix} 15614.69 & 17691.57 & 19773.95 \\ 17691.57 & 20366.48 & 23124.19 \\ 19773.95 & 23124.19 & 26652.79 \end{bmatrix}$$

The next step is to find the eigenvalues and eigenvectors for this matrix.

As this data proved complicated to factorise by hand, an alternate matrix has been included below, to demonstrate the technique.

For this example:

$$\text{Matrix A} = \begin{bmatrix} 3 & 1 & -1 \\ 1 & 3 & -1 \\ -1 & -1 & 5 \end{bmatrix}$$

To find the Eigenvalues:

The first step is to find the determinant $(A - \lambda I)$:

So $\begin{bmatrix} 3-\lambda & 1 & -1 \\ 1 & 3-\lambda & -1 \\ -1 & -1 & 5-\lambda \end{bmatrix}$

$= (3-\lambda) \begin{vmatrix} 3-\lambda & -1 \\ -1 & 5-\lambda \end{vmatrix} - 1 \begin{vmatrix} 1 & -1 \\ -1 & 5-\lambda \end{vmatrix} - 1 \begin{vmatrix} 1 & 3-\lambda \\ -1 & -1 \end{vmatrix}$

$= (3-\lambda) \left((3-\lambda)(5-\lambda) - (-1)(-1)\right) - 1 \left(1(5-\lambda) - (-1)(-1)\right) - \left(1(-1) - (-1)(3-\lambda)\right)$

Simplify:

$= (3-\lambda)(15 - 3\lambda - 5\lambda + \lambda^2 - 1) - 1(5 - \lambda - 1) - 1(-1 + 3 - \lambda)$

$= (3-\lambda)(\lambda^2 - 8\lambda + 14) - (-\lambda + 4) - (-\lambda + 2)$

Multiply:

$= 3\lambda^2 - 24\lambda + 42 - \lambda^3 + 8\lambda^2 - 14\lambda + \lambda - 4 + \lambda - 2$

Rearrange:

$= -\lambda^3 + 11\lambda^2 - 36\lambda + 36$

Factorise: $a\overline{)\,c}^{\,b}$ and $a \times b = c$

Factorise:

$$
\begin{array}{r|l}
 & -\lambda^2 + 9\lambda - 18 \\
\hline
\lambda - 2 & -\lambda^3 + 11\lambda^2 - 36\lambda + 36 \\
 & -(-\lambda^3 + 2\lambda^2) \\
\hline
 & 0 + 9\lambda^2 - 36\lambda + 36 \\
 & 9\lambda^2 - 18\lambda \\
\hline
 & 0 - 18\lambda + 36 \\
 & -(-18\lambda + 36) \\
\hline
 & 0
\end{array}
$$

So,

$-\lambda^3 + 11\lambda^2 - 36\lambda + 36$

$= (\lambda-2)(-\lambda^2 + 9\lambda - 18)$

$= (\lambda-2)(-\lambda + 3)(\lambda - 6) = 0$

As each bracket = 0;

$\lambda-2 = 0$ Therefore $\lambda = 2$

$-\lambda+3 = 0$ Therefore $\lambda = 3$ } These are the eigenvalues.

$\lambda-6 = 0$ Therefore $\lambda = 6$

The eigenvectors are calculated for each of the eigenvalues.

a. For $\lambda = 2$

$(A - \lambda_1 I)\,\underline{v}_1 = 0$

$$\underline{v}_1 = \begin{pmatrix} v_{1,1} \\ v_{1,2} \\ v_{1,3} \end{pmatrix}$$

$$\begin{pmatrix} 3-\lambda & 1 & -1 \\ 1 & 3-\lambda & -1 \\ -1 & -1 & 5-\lambda \end{pmatrix} = \begin{pmatrix} 3-2 & 1 & -1 \\ 1 & 3-2 & -1 \\ -1 & -1 & 5-2 \end{pmatrix} = \begin{pmatrix} 1 & 1 & -1 \\ 1 & 1 & -1 \\ -1 & -1 & 3 \end{pmatrix}$$

$$\begin{pmatrix} 1 & 1 & -1 \\ 1 & 1 & -1 \\ -1 & -1 & 3 \end{pmatrix} \times \begin{pmatrix} v_{1,1} \\ v_{1,2} \\ v_{1,3} \end{pmatrix} = 0$$

$$\begin{pmatrix} v_{1,1} + v_{1,2} - v_{1,3} \\ v_{1,1} + v_{1,2} - v_{1,3} \\ -v_{1,1} - v_{1,2} + 3v_{1,3} \end{pmatrix} = 0 \qquad \text{So, each row} = 0$$

$v_{1,1} + v_{1,2} - v_{1,3} = 0$

$-v_{1,1} - v_{1,2} + 3v_{1,3} = 0$

Rearrange to find $V_{1,3}$:

$v_{1,3} = v_{1,1} + v_{1,2} \Rightarrow 3v_{1,3} = 3v_{1,1} + 3v_{1,2}$

Replace this value of $3V_{1,3}$ into the second equation:

$-v_{1,1} - v_{1,2} + 3v_{1,1} + 3v_{1,2} = 0$

$2v_{1,1} + 2v_{1,2} = 0$

$v_{1,1} = -v_{1,2}$

So, $v_{1,1} = \begin{pmatrix} 1 \\ -1 \\ 0 \end{pmatrix}$ (Because there is no $v_{1,3}$ in the final equation.

This is the eigenvector for $\lambda = 2$.

b. For $\lambda = 3$

$(A - \lambda_2 I)\ \underline{v}_2 = 0$

$$\underline{V}_2 = \begin{pmatrix} v_{2,1} \\ v_{2,2} \\ v_{2,3} \end{pmatrix}$$

$$\begin{pmatrix} 3-\lambda & 1 & -1 \\ 1 & 3-\lambda & -1 \\ -1 & -1 & 5-\lambda \end{pmatrix} = \begin{pmatrix} 3-3 & 1 & -1 \\ 1 & 3-3 & -1 \\ -1 & -1 & 5-3 \end{pmatrix} = \begin{pmatrix} 0 & 1 & -1 \\ 1 & 0 & -1 \\ -1 & -1 & 2 \end{pmatrix}$$

$$\begin{pmatrix} 0 & 1 & -1 \\ 1 & 0 & -1 \\ -1 & -1 & 2 \end{pmatrix} \times \begin{pmatrix} v_{2,1} \\ v_{2,2} \\ v_{2,3} \end{pmatrix} = 0$$

$$\begin{pmatrix} v_{2,2} - v_{2,3} \\ v_{2,1} - v_{2,3} \\ -v_{2,1} - v_{2,2} + 2v_{2,3} \end{pmatrix} = 0$$ So, each row = 0

$v_{2,2} - v_{2,3} = 0$ So, $v_{2,2} = v_{2,3}$

$v_{2,1} - v_{2,3} = 0$ So, $v_{2,1} = v_{2,3}$

$-v_{2,1} - v_{2,2} + 2v_{2,3} = 0$ So, $v_{2,1}$, $v_{2,2}$ and $v_{2,3}$ are equal.

So, $$v_{2,1} = \begin{pmatrix} 1 \\ 1 \\ 1 \end{pmatrix}$$

$$V_{2,2} = \begin{pmatrix} 1 \\ 1 \\ 1 \end{pmatrix}$$

$$V_{2,3} = \begin{pmatrix} 1 \\ 1 \\ 1 \end{pmatrix}$$

This is the eigenvector for $\lambda = 3$.

c. For $\lambda = 6$

$(A - \lambda_3 I)\,\underline{v}_3 = 0$

$$\underline{v}_3 = \begin{pmatrix} v_{3,1} \\ v_{3,2} \\ v_{3,3} \end{pmatrix}$$

$$\begin{pmatrix} 3-\lambda & 1 & -1 \\ 1 & 3-\lambda & -1 \\ -1 & -1 & 5-\lambda \end{pmatrix} = \begin{pmatrix} 3-6 & 1 & -1 \\ 1 & 3-6 & -1 \\ -1 & -1 & 5-6 \end{pmatrix} = \begin{pmatrix} -3 & 1 & -1 \\ 1 & -3 & -1 \\ -1 & -1 & -1 \end{pmatrix}$$

$$\begin{pmatrix} -3 & 1 & -1 \\ 1 & -3 & -1 \\ -1 & -1 & -1 \end{pmatrix} \text{ x } \begin{pmatrix} v_{3,1} \\ v_{3,2} \\ v_{3,3} \end{pmatrix} = 0$$

$$\begin{pmatrix} -3v_{3,1} + v_{3,2} - v_{3,3} \\ v_{3,1} - 3v_{3,2} - v_{3,3} \\ -v_{3,1} - v_{3,2} + v_{3,3} \end{pmatrix} = 0 \quad \text{So, each row} = 0$$

$-3v_{3,1} + v_{3,2} - v_{3,3} = 0$ (equation 1)

$v_{3,1} - 3v_{3,2} - v_{3,3} = 0$ (equation 2)

$-v_{3,1} - v_{3,2} - v_{3,3} = 0$ (equation 3)

To remove v_3, subtract equation 2 from equation 1.

$-3v_{3,1} + v_{3,2} - v_{3,3} - v_{3,1} + 3v_{3,2} + v_{3,3} = 0$

$-4v_{3,1} + 4v_{3,2} = 0$

$v_{3,1} = v_{3,2}$

Then,

$-v_{3,1} - v_{3,2} - v_{3,3} = 0$

And $-v_{3,1} - v_{3,1} - v_{3,3} = 0$

And $-2v_{3,1} = v_{3,3}$

So, $v_{3,1} = \begin{pmatrix} 1 \\ 1 \\ -2 \end{pmatrix}$

$V_{3,2} =$

$V_{3,3} =$

This is the eigenvector for $\lambda = 6$.

An eigenvector gives the position on a multidimensional graph, where each one represents a position of a different axis. The more values present represents a greater number of dimensions.

Once the eigenvalues and corresponding eigenvectors have been calculated, they are lined up from highest to lowest in order to interpret the results. Eigenvector 1 is the first principal component because it has the highest eigenvalue.

The eigenvalues for the small sample of my data was calculated using data points originating from DSC thermograms, which plot heat flow against temperature. The results were as follows:

$\lambda 1 \approx 62016.6$ This is the first principal component because it is the highest value.

$\lambda 2 \approx 617.344$ This is the second principal component.

$\lambda 3 \approx 0.005$ This is the third principal component.

Corresponding eigenvectors:

Principal Component

$v_1 \approx \begin{pmatrix} -0.496372 \\ -0.57294 \\ -0.652192 \end{pmatrix}$ The vector is closest to this axis, therefore this is the most important for influencing the results.

1

$v_2 \approx \begin{pmatrix} -0.736324 \\ -0.120077 \\ 0.665889 \end{pmatrix}$

2

$v_3 \approx \begin{pmatrix} 0.459828 \\ -0.810754 \\ 0.362266 \end{pmatrix}$

3

To find the variation in the data:

In this case $\frac{\lambda}{\lambda_1+\lambda_2+\lambda_3}$ = 0.990% for component 1.

Linear Discriminant Analysis

The data used for this analysis was based on the unweighted data and used the first five principal components. The first five were used as they encompassed 90.6% of the variance between the cases. The same technique would be applied for the weighted data.

	Type	PC1	PC2	PC3	PC4	PC5
Case16	Unknown	1.611569	9.979493	22.02392	10.77099	10.96247
Case24	Unknown	9.30618	-1.17388	37.34808	16.02456	18.13521
Case30	Unknown	-27.4899	6.732931	8.849905	8.032669	4.403203
Case74	Unknown	59.61188	-0.21113	2.560188	1.207882	10.65152
Case79	Unknown	33.58785	-8.32657	-7.91726	3.51313	11.31877
Case82	Unknown	17.74199	5.162663	-9.31653	12.09781	-29.5744
Case83	Unknown	73.66236	-15.6356	5.012886	3.532008	11.71255
Case86	Unknown	101.3126	-9.69608	15.58579	4.896815	11.58848
MA061	Pharma	-40.7589	-0.80465	-4.56029	9.020978	2.772301
MA061-19	Pharma	-107.293	-29.9489	-10.5968	16.95552	0.373502
MA061-21	Pharma	-47.6988	-20.8416	3.189416	13.45571	-14.651
MA061-23	Pharma	-39.6191	-21.3693	8.729064	6.896403	-17.7891
MA064-11	Pharma	-47.0201	-26.1642	7.907796	8.528296	-8.45288
MA064-14	Pharma	-89.5017	-26.451	4.618471	13.21014	-9.03729
MA064-17	Pharma	-48.8645	-29.6766	10.07736	17.58832	-9.7547
MA064-20	Pharma	-47.8942	-25.7434	9.80379	11.88389	-15.5551

The first step was to calculate the average of the 8 principal component values for each type of case (Sayad, 2017).

The results of the calculation are presented in the Principal Component Analysis Data Table below.

Type	Count (*N*)	Statistics	PC1	PC2	PC3	PC4	PC5
Pharmaceutical	8	Mean (μ1)	-58.58	-22.63	3.65	12.19	-9.01
Unknown	8	Mean (μ2)	33.67	-1.65	9.27	7.51	6.15

The calculation for the covariant matrices results in a 5x5 matrix for each type. The layout shows the variance on the diagonal and covariance to complete the remaining entries. This layout is demonstrated in the matrix layout below (Berman, 2017).

Variance	Covariance	Covariance	Covariance	Covariance
Covariance	Variance	Covariance	Covariance	Covariance
Covariance	Covariance	Variance	Covariance	Covariance
Covariance	Covariance	Covariance	Variance	Covariance
Covariance	Covariance	Covariance	Covariance	Variance

In order to visualise the positions of the entries described below, the positions of the entries are demonstrated in the following table.

1	2	3	4	5
6	7	8	9	10
11	12	13	14	15
16	17	18	19	20
21	22	23	24	25

As the table is reflective on the diagonal, only half the table needs to be calculated and copied over.

Each variance calculation is the same but the value of x varies according to its position in the matrix. Each covariance calculation varies in the same way. For example, to find the value of position 9 in row 2 and column 4, the values used would be x_2 and x_4 (Berman, 2017).

The calculation for entries 1 and 2 would be slightly different as one is for variance and the other covariance. The equations for each are shown below (Columbia Business School, 2003).

Entry 1 (variance) would be calculated using the equation:

$$\sum_{cases}(x_1 - \overline{x_1})^2/(N-1)$$

$$\sum_{cases}(PC1\ value - \overline{PC1})^2/(N-1)$$

= (Case MA061 PC1 value – average of PC1)^2 /(N-1)

+ (Case MA061-19 PC1 value – average of PC1)^2 /(N-1)

+ (Case MA061-21 PC1 value – average of PC1)^2 /(N-1)

+ (Case MA061-23 PC1 value – average of PC1)^2 /(N-1)

+ (Case MA064-11 PC1 value – average of PC1)^2 /(N-1)

+ (Case MA064-14 PC1 value – average of PC1)^2 /(N-1)

+ (Case MA064-17 PC1 value – average of PC1)^2 /(N-1)

+ (Case MA064-20 PC1 value – average of PC1)^2 /(N-1)

= (-40.76 - (-58.58))^2 / 7 + (-107.29 - (-58.58))^2 / 7 + (-47.70 - (58.58))^2 / 7

+ (-39.63 - (-58.58))^2 / 7 + (-47.02 - (-58.58))^2 / 7 + (-89.50 - (58.58))^2 / 7

+ (-48.86 - (-58.58))^2 / 7 + (-47.89 - (58.58))^2 / 7

= 45.38 + 338.98 + 16.92 + 51.37 + 19.09 + 136.58 + 13.49 + 16.32

= 638.12

<u>The Calculation for entry 2</u> (row 1 column 2) is for covariance and uses the equation:

$$\sum_{cases} (x_1 - \overline{x_1})(x_2 - \overline{x_2}) \,/\, (N-1)$$

$$\sum_{cases} (PC1\ value - \overline{PC1})\ (PC2\ value - \overline{PC2})\ /\ (N-1)$$

= (Case MA061 PC1 value – average of PC1) x (Case MA061 PC2 – average of PC2) /(N-1)

+ (Case MA061-19 PC1 value – average of PC1) x (Case MA061-19 PC2 – average of PC2) /(N-1)

+ (Case MA061-21 PC1 value – average of PC1) x (Case MA061-21 PC2 – average of PC2) /(N-1)

+ (Case MA061-23 PC1 value – average of PC1) x (Case MA061-23 PC2 – average of PC2) /(N-1)

+ (Case MA064-11 PC1 value – average of PC1) x (Case MA064-11 PC2 – average of PC2) /(N-1)

+ (Case MA064-14 PC1 value – average of PC1) x (Case MA064-14 PC2 – average of PC2) /(N-1)

+ (Case MA064-17 PC1 value – average of PC1) x (Case MA064-17 PC2 – average of PC2) /(N-1)

+ (Case MA064-20 PC1 value – average of PC1) x (Case MA064-20 PC2 – average of PC2) /(N-1)

=

(-40.76 – (-58.58)) x (-0.80 – (22.63)) / 7 + (-107.293 – (-58.58)) x (-29.95 –

(-22.63)) / 7

+ (-47.70 – (-58.58)) x (-20.84 – (22.63)) / 7 + (-39.62 – (-58.58)) x (-21.37 –

(-22.63)) / 7

+ (-47.02 – (-58.58)) x (-26.16 – (22.63)) / 7 + (-89.50 – (-58.58)) x (-26.45 –

(-22.63)) / 7

+ (-48.86 – (-58.58)) x (-29.68 – (22.63)) / 7 + (-47.89 – (-58.58)) x (-25.74 –

(-22.63)) / 7

= 109.20

The resulting covariance matrix for the pharmaceutical cases would be:

$$C_1 = \begin{pmatrix} 638.12 & 109.20 & 112.35 & -57.13 & -77.84 \\ 109.20 & 88.73 & -21.79 & -19.50 & 27.30 \\ 112.35 & -21.79 & 56.38 & -7.30 & -45.02 \\ -57.13 & -19.50 & -7.30 & 15.15 & 6.09 \\ -77.84 & 27.30 & -45.02 & 6.09 & 54.21 \end{pmatrix}$$

The resulting covariance matrix for the unknown cases would be:

$$C_2 = \begin{pmatrix} 1780.50 & -295.91 & -98.13 & -130.86 & 133.75 \\ -295.91 & 79.88 & 20.77 & 24.56 & -49.42 \\ -98.13 & 20.77 & 242.16 & 43.80 & 136.32 \\ -130.86 & 24.56 & 43.80 & 26.11 & -18.92 \\ 133.75 & -49.42 & 136.32 & -18.92 & 221.94 \end{pmatrix}$$

From these a pooled covariance matrix is calculated (Sayad, 2017):

$$C = \frac{1}{N_1 + N_2}(N_1 C_1 + N_2 C_2)$$

$$= \frac{1}{8 + 8}(8C_1 + 8C_2)$$

$$= \frac{1}{2}(C_1 + C_2)$$

$$C = \begin{pmatrix} 1209.31 & -93.35 & 7.11 & -94.00 & 27.96 \\ -93.35 & 84.31 & -0.51 & 2.53 & -11.06 \\ 7.11 & -0.51 & 149.27 & 18.25 & 45.65 \\ -94.00 & 2.53 & 18.25 & 20.63 & -6.41 \\ 27.96 & -11.06 & 45.65 & -6.41 & 138.08 \end{pmatrix}$$

This pooled covariance matrix then needs to be inverted.

This is demonstrated below using more simple data.

$$\begin{pmatrix} 1 & 4 & 4 \\ 1 & 2 & 4 \\ 1 & 3 & 2 \end{pmatrix}$$

Elementary row operations are conducted to turn this into a 3x3 identity matrix. Every operation used to create this is also performed on a separate 3x3 identity matrix to produce the inverse of the above matrix.

$$\begin{matrix} R_1 \\ R_2 \\ R_3 \end{matrix} \begin{pmatrix} 1 & 4 & 4 \\ 1 & 2 & 4 \\ 1 & 3 & 2 \end{pmatrix} \qquad \begin{pmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{pmatrix}$$

$R_2 \rightarrow R_2 - R_1$

$R_3 \rightarrow R_3 - R_1$

$$\begin{pmatrix} 1 & 4 & 4 \\ 0 & -2 & 0 \\ 0 & -1 & -2 \end{pmatrix} \qquad \begin{pmatrix} 1 & 0 & 0 \\ -1 & 1 & 0 \\ -1 & 0 & 1 \end{pmatrix}$$

$R_2 \rightarrow - R_2/2$

$$\begin{pmatrix} 1 & 4 & 4 \\ 0 & 1 & 0 \\ 0 & -1 & -2 \end{pmatrix} \qquad \begin{pmatrix} 1 & 0 & 0 \\ 0.5 & -0.5 & 0 \\ -1 & 0 & 1 \end{pmatrix}$$

$R_1 \rightarrow R_1 - 4R_2$

$R_3 \quad R_3 + R_2$

$$\begin{pmatrix} 1 & 0 & 4 \\ 0 & 1 & 0 \\ 0 & 0 & -2 \end{pmatrix} \qquad \begin{pmatrix} -1 & 2 & 0 \\ 0.5 & -0.5 & 0 \\ -0.5 & -0.5 & 1 \end{pmatrix}$$

$R_3 \rightarrow - R_3/2$

$$\begin{pmatrix} 1 & 0 & 4 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{pmatrix} \qquad \begin{pmatrix} -1 & 2 & 0 \\ 0.5 & -0.5 & 0 \\ 0.25 & 0.25 & -0.5 \end{pmatrix}$$

$R_1 \rightarrow R_1 - 4R_3$

$$\begin{pmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{pmatrix} \quad \begin{pmatrix} -2 & 1 & 2 \\ 0.5 & -0.5 & 0 \\ 0.25 & 0.25 & -0.5 \end{pmatrix}$$

Returning to the unweighted data, the information was inserted into: http://matrix.reshish.com/inverCalculation.php (Reshish.com, 2017).

The results showed the inverted matrix would be:

$$C^{-1} = \begin{pmatrix} 0.001597 & 0.001589 & -0.00129 & 0.08415 & 0.000621 \\ 0.001589 & 0.013599 & -0.0014 & 0.007302 & 0.00157 \\ -0.00129 & -0.0014 & 0.009832 & -0.01559 & -0.00383 \\ 0.08415 & 0.007302 & -0.01559 & 0.102441 & 0.00879 \\ 0.000621 & 0.00157 & -0.00383 & 0.00879 & 0.008915 \end{pmatrix}$$

The coefficients can then be calculated. (Sayad, 2017).

$$C^{-1}(-\mu_1 + \mu_2)$$

$$= C^{-1}\left(-\begin{pmatrix} -58.58 \\ -22.63 \\ 3.65 \\ 12.19 \\ -9.01 \end{pmatrix} + \begin{pmatrix} 33.67 \\ -1.65 \\ 9.27 \\ 7.51 \\ 6.15 \end{pmatrix} \right)$$

$$= C^{-1}\begin{pmatrix} 92.25 \\ 20.98 \\ 5.63 \\ -4.68 \\ 15.16 \end{pmatrix}$$

$$= [0.143413 \quad 0.413628 \quad -0.07807 \quad 0.495355 \quad 0.167]$$

‘R’ Scripts used for PCA Analysis

options(max.print=999999) #(This increases the number of print lines in R because the file is big)

MAset<-read.table("MA tablets_22Feb17.txt", header =TRUE) #(Read the table in from the file and store as object ‘MAset’)

comp=prcomp(MAset, scale = TRUE) # (Run the pca on the dataset MAset using a built in function called prcomp and assign outcome to a variable ‘comp’. TRUE means that the data are standardised so that each variable has a mean=0 and a standard deviation =1)

summary(comp) # (Get a summary of the outcome of the PCA - output of 34 components).

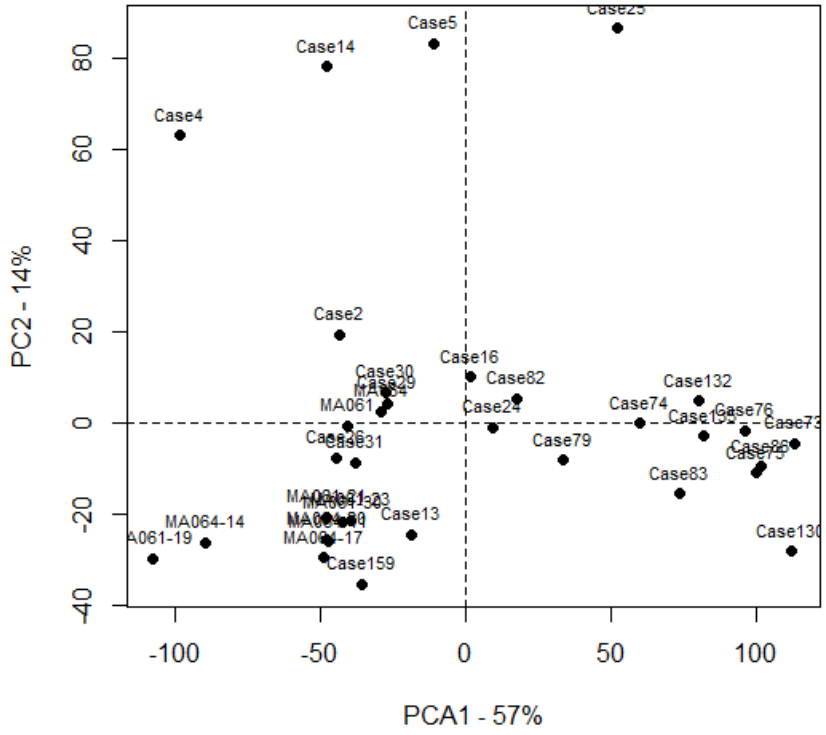
compvalue<-comp$x # (To view the component value for each observation in comp. Assign this output to ‘compvalue’).

compvalue # (Print out compvalue. This gives you all the principal components usually far more than needed).
(output of observation values across 34 components).

To Plot A Bi-Plot, there are 3 separate steps most of the code relates to axes labels and choice of dot.

plot(compvalue[,1], compvalue[,2], pch=19, xlab = "PCA1 - 57%", ylab ="PC2 - 14%") # (plotting the observations on the first two principal components)

abline(h=0, v=0, lty =2) # (put in the horizontal and vertical dotted lines)
text(compvalue[,1], compvalue[,2], labels = rownames(compvalue), cex=0.7, pos=3)



WEIGHTINGS

out=comp$rotation (To obtain the weightings of the individual variables to each component)

write.csv(out, file="loadings2.txt", col.names=TRUE, row.names=TRUE) (To write the loadings to text file, there are too many for the screen)

write.csv(compvalue, file="MAcompvalue_Mae.txt", col.names=TRUE, row.names=TRUE)

Appendix II – Accepted Ethics Form

Form E1 2013 v10.2

Form E1

Research – Ethical Considerations **School Of Science, Engineering & Technology**

This form must be completed for all research projects: undergraduate, postgraduate and staff. Following submission to an Ethics Rep (see page 7), the form will be returned to the Proposer/Supervisor, indicating the outcome (see pages 8-9).

This form must be completed electronically and submitted by email.
To enter information, please tab between the grey field boxes.

Responsibilities

- Where the **Proposer** is STAFF: completion of this form and submission to Ethics Rep.
- Where the **Proposer** is a STUDENT (conducting a project within the University): completion of this form and submission to Supervisor or designated programme contact[1].
- Where the **Proposer** is a PLACEMENT STUDENT (conducting an EXTERNAL project, outwith the University): completion of this form and submission to designated programme contact[2].
- **Supervisor:** check and complete student's form and submit to Ethics Rep.

HELP NOTES: where applicable, important help notes are provided in the Word status bar (at the foot of your screen) when you move to an entry field. Please look out for these notes.

Section A General Information

A1 Name of Project Proposer:
M. MacDougall-Heasman

A2 Matriculation No. (where applicable):
1001227

A3 Programme (where applicable):
Research Studentship

A4 Module Code/Title (where applicable):

A5 Supervisor (if not the Proposer):
Isobel Stewart

A6 Title of Project:
The Chemical and Physical Characterisation of Illicit Tablets and Development of a Statistical Model

A7 Main aim of study (max. 50 words):

To provide drug intelligence information enabling the police to determine links between different seizures, through measurement of physical & chemical characteristics and data analysis

[1] Some programmes may designate an alternative person (e.g. project module tutor) as the recipient of completed E1 forms. The Programme Tutor concerned should be consulted if in doubt.
[2] Note that for external projects the completed E1 form should be forwarded to the designated recipient (as advised by the Programme Tutor concerned); the form should NOT normally be forwarded to either the internal (University) Supervisor or the external (placement) Supervisor.

A8 Site of the Research:
University of Abertay, Dundee

A9 For external projects

i.e. projects conducted fully or partly outwith Abertay University

A9.1 Name of external local ethical approval body:

A9.2 Local ethical application status: Approved ☐ Pending ☐ Declined ☐

A9.3 Reference Date (dd/mm/yy)

Please note that, in the case of external projects, both external ethical approval and University approval are required.

A10 Outline of Research Proposal

Please supply a brief outline of the research proposal in the field below (max. 200 words).

- Ensure you highlight any areas of potential ethical relevance.
- If you consider there to be no areas of ethical relevance, please make this clear.

There is a gap in drugs intelligence knowledge at local, national and international levels. Analytical data that better characterises illicit drugs, could assist police and scientists when preparing to give evidence of opinion and may help to establish a starting point in evidentially linking seizures and criminal groups in illicit drug production. Scientific information will also highlight any significant variation in illicit preparation potency relevant to health care professionals.

This project will study two different illicit table preparations: diazepam and ecstasy and will investigate the process and degradation impurities resulting from the drug synthesis. It will examine the adulterants, cutting agents, binders and compression into tablets which result from the manufacturing process.

Certain physical characteristics including colour, diameter, imprint details, surface morphology and colour distribution will also be assessed.

Resulting information and data will be analysed with multivariate statistical techniques to identify clusters within the measured variables and relate these to specific groups of tablets.

Ethics of the project concerns the proper care and analysis of drugs under investigation. Crown Office approval has been given to obtain samples of street drugs from closed cases for the purposes of this project. The School of Science, Engineering and Technology are in possession of the required Home Office drug licence.

Section B Ethical Aspects

#	Please answer the following questions:	YES	NO
B1	Does a significant risk of **physical or psychological harm** exist (following Risk Assessment and control measures)?	☐	☒
B2	Could the research harm the **reputation** of the University?	☐	☒
B3	Will the research involve **animals** of a type requiring a Home Office licence?	☐	☒
B4	Will the research involve **genetic modification** (GM)?	☐	☒
B5	Will the research involve **stored human samples**, for example organs, tissues, cells (excluding established cell lines)?	☐	☒
B6	Will the research involve **human subjects** in any manner? (e.g. as experimental subjects, or as interviewees, or as the source of biological materials)	☐	☒
B7	Will the research involve the use of **computers** or the **network** for other than an IT function i.e. for more than the use of applications such as MS Word or Excel to write a report or analyse data?	☐	☒

Re:	**Action Taken** Where any of B1-B6 above have been marked "yes", the following action is required as applicable:	*Tick to confirm action taken*
B1	Supply a copy of the Risk Assessment[3]	☐
B2	In Section C (page 4), explain how reputation risks will be minimised	☐
B3	Append a copy of the Home Office licence (or, in the case of a pending decision, append a copy of the Home Office application)[6]	☐
B4	Append a copy of GMOC approval, (or, in the case of a pending decision, append a copy of the GMOC application)[6]	☐
B5	Explain in Section C (page 4) how the human material will be employed and handled in accordance with the relevant legislation	☐
B6	Complete Section D (page 5)	☐
B7	In Section C (page 4), explain how you will ensure compliance with relevant legislation on computer misuse and data protection.	☐
Where <u>none</u> of B1-B6 above have been marked "yes", please turn straight to Section E (page 7)		

[3] In the case of external projects for which this requirement has been fulfilled by the external project provider concerned, it will suffice to supply reference information only (normally comprising title, number, date); a full copy of the external documentation is <u>not</u> required. Enter such information into section C.

Section C Further information as indicated in Section B (if applicable)
(max. 200 words)

Section D Research involving human subjects (if applicable)

D1 Number of human research subjects (approx):

D2 Who are the research subjects? (Please state inclusion/exclusion criteria):

D3 How will you recruit them for your study?

D4 Research Approach & Procedures

	Yes
Qualitative	☐
Survey	☐
Experiment	☐
Literature Based	☐
Questionnaires	☐
Questionnaires supplied with this form (in a separate file)	☐
Interviews	☐
Observation – overt	☐
Observation - covert (specify in box below)	☐
Other research procedures being used (specify in box below)	☐

D5 Could any of these procedures cause discomfort, anxiety, stress or embarrassment?

Yes ☐ No ☐

(D5, continued) If the answer to D5 is yes, explain how you will seek to minimise the impact:

D6 Explain the procedures you will employ to ensure informed consent and confidentiality:

D7 Precautions and safeguards (please answer all questions):

	Yes
D7.1 Will the subject be provided with a written/oral explanation of the project?	☐
D7.2 Will the research subjects be asked to fill in a consent form?	☐
D7.3 Will it be explained to the research subjects that they may not benefit from your study?	☐
D7.4 Will the research subjects be offered the opportunity to decline to take part?	☐
D7.5 Will the research subjects be offered the opportunity to withdraw at any stage?	☐
D7.6 Will a guarantee of confidentiality be expressed to the subjects?	☐
D7.7 Will an offer of anonymity be given to the subjects?	☐
D7.8 Will the provisions of the Data Protection Act be adhered to?	☐
D7.9 Will the subjects be briefed on health and safety issues and any risks involved?	☐
D7.10 Will the subjects be provided with written contact details of a member of the research team?	☐

If any of the questions in D7 cannot be answered in the affirmative, provide an explanation:

Section E Confirmation

E1 I have read the *School Research Ethics Committee's Principles & Procedures document,* the *School Health & Safety Policy* and the *University of Abertay Dundee Health & Safety Policy* (available on the Portal) and will adhere to these policies

Yes ☒

E2 I understand that it is my responsibility to ensure compliance with any relevant regulatory or legal requirements (such as data protection legislation, stored tissue regulations, animal experimentation licensing, etc)

Yes ☒

E3 This form has been prepared by the Proposer (as named in section A1)

Yes ☒

E4 This form has been read and approved by the Supervisor (as named in section A5)

Yes ☒ Not applicable ☐

Section F Steps to finalise & send this form

1. Student Proposers (where applicable) - send this to your Supervisor
2. Staff Proposers / Supervisors - please choose the most appropriate rep for the research activity concerned:

   Mr D Phillips - Forensic Science projects

Then please email this form to: **set@abertay.ac.uk**

The outcome of the application will be indicated on pages 8 onwards when the final E1 is emailed back to the Proposer / Supervisor.

Important: research must not commence until ethical approval has been recommended.

Section G ***For completion by the Ethics Rep***

G1 Completed proposal form received by Ethics Representative

Mr D Phillips - Forensic Science projects

Date received (dd/mm/yy) 04/04/14

Note: where an ethically contentious project is proposed, it is good practice for the Ethics Rep to ask a second Rep (or the SREC Chair) to take an independent look at the E1 form. Examples of ethically contentious projects include those involving vulnerable groups such as children, or where a significant risk of harm (psychological or physical) or reputational risk exists.

G2 The following recommendation(s) have been made:

G2.1 The project is satisfactory and should proceed	☒
G2.2 The project should proceed on condition that all questionnaires, surveys and communications with external persons or bodies are discussed and approved in advance with the Project Supervisor.	☐
G2.3 The project should proceed on condition that the requirements set in the box below are complied with	☐

Where the recommendation is G2.1, G2.2 or G2.3 (above), the research project may commence. (Homologation will be made subsequently by the SREC.)

G3 The proposal requires to be re-submitted (to the ethics rep) with additional information regarding:	☐
G3.1 Organisms and / or subjects (delete as appropriate)	☐
G3.2 Methods of data collection/procedures	☐
G3.3 Informed Consent	☐
G3.4 Confidentiality	☐
G3.5 Other (as per the box below)	☐

(G3.5, continued)

G4 The Project described in the Proposal has been considered not suitable for research, on the grounds of:	☐
G4.1 Unacceptable harm to organisms and / or subjects	☐
G4.2 Potential harm to the researcher	☐
G4.3 Risk of bringing the University into disrepute.	☐
G4.4 Other (as per the box below)	☐
You are recommended to submit an alternative proposal.	

G5 The Proposal will be considered by the SREC Chair for possible submission to a full meeting of the School Research Ethics Committee; the outcome will be relayed to you in due course.	☐

G6 The Proposal will be considered by the SREC Chair for possible scrutiny by an independent ethics rep in another School; the outcome will be relayed to you in due course.	☐

Where the recommendation is G3, G4, G5 or G6 (above), research must not commence until ethical approval has been given.

For attention of undergraduate & MSc students: the recommendations recorded in this from should be discussed with your Project Supervisor, not with the ethics rep.

Ethics rep actions

The rep should now email this form back to set@abertay.ac.uk

School Office Actions

(I) Following receipt of this form from the Proposer/Supervisor:

(Ia) Key details to be recorded on the electronic log	☒
(Ib) Form to be emailed to the rep named by the Proposer, or to an alternative rep if the named rep is unavailable	☒

(II) Following receipt of this form (complete with decision) from the Rep:

(IIa) Update electronic log with decision details	☒
(IIb) Retain an electronic copy of the form for records	☒
(IIc) Email a copy of the form to the Proposer (in the case of a staff project) or to the Supervisor (in the case of a student project)	☒

Additionally:

Undergraduate and MSc project proposals should be periodically checked-off against the OASIS list of project module registrants, and a summary sent to the SREC Chair. This could occur approximately weekly during the first 4 weeks of each semester, and approximately fortnightly for the reminder of the semester.

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